ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
UNIVERSITY OF CRETE

ΙΑΤΡΙΚΗ ΣΧΟΛΗ
SCHOOL OF MEDICINE

ΕΡΓΑΣΤΗΡΙΟ ΑΝΑΤΟΜΙΑΣ

ΔΙΕΥΘΥΝΤΗΣ: ΑΝΑΠΛΗΡΩΤΗΣ ΚΑΘΗΓΗΤΗΣ ΙΩΑΝΝΗΣ ΤΣΙΑΟΥΣΗΣ

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ΤΑΞΙΑΡΧΗΣ ΚΩΝΣΤΑΝΤΙΝΟΣ ΝΙΚΟΛΟΥΖΑΚΗΣ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

ΗΡΑΚΛΕΙΟ, 2021

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
UNIVERSITY OF CRETE

ΙΑΤΡΙΚΗ ΣΧΟΛΗ
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HERAKLION, 2021

ΑΝΙΧΝΕΥΣΗ ΠΡΟΓΝΩΣΤΙΚΩΝ ΒΙΟΛΟΓΙΚΩΝ ΔΕΙΚΤΩΝ ΣΤΟ ΜΕΤΑΣΤΑΤΙΚΟ ΚΑΡΚΙΝΟ ΤΟΥ ΠΑΧΕΟΣ ΕΝΤΕΡΟΥ ΚΑΙ ΤΟΠΙΚΩΣ ΠΡΟΧΩΡΗΜΕΝΟ ΚΑΡΚΙΝΟ ΤΟΥ ΟΡΘΟΥ

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

Υποβληθείσα στην Ιατρική Σχολή

του

Πανεπιστημίου Κρήτης

Υπό του

ΤΑΞΙΑΡΧΗ ΚΩΝΣΤΑΝΤΙΝΟΥ ΝΙΚΟΛΟΥΖΑΚΗ

Για την απόκτηση του τίτλου του Διδάκτορα της

Ιατρικής Σχολής

του Πανεπιστημίου Κρήτης

Ηράκλειο 2021

ΑΝΙΧΝΕΥΣΗ ΠΡΟΓΝΩΣΤΙΚΩΝ ΒΙΟΛΟΓΙΚΩΝ ΔΕΙΚΤΩΝ ΣΤΟ ΜΕΤΑΣΤΑΤΙΚΟ ΚΑΡΚΙΝΟ ΤΟΥ ΠΑΧΕΟΣ ΕΝΤΕΡΟΥ ΚΑΙ ΤΟΠΙΚΩΣ ΠΡΟΧΩΡΗΜΕΝΟ ΚΑΡΚΙΝΟ ΤΟΥ ΟΡΘΟΥ

Υπό του

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Ιατρική Σχολή

Πανεπιστημίου Κρήτης

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Γεώργιος Γουλιέλμος	Καθηγητής Γενετικής Ιατρικής Σχολής Πανεπιστημίου Κρήτης
Eelco de Bree	Καθηγητής Γενικής Χειρουργικής - Χειρουργικής Ογκολογίας Πανεπιστημίου Κρήτης
Εμμανουήλ Χρυσός	Καθηγητής Χειρουργικής Ιατρικής Σχολής Πανεπιστημίου Κρήτης

Η ΕΞΕΤΑΣΤΙΚΗ ΕΠΙΤΡΟΠΗ

Ιωάννης Τσιαούσης

Ιωάννης Σουγκλάκος

Αριστείδης Τσατσάκης

Μαρία Τζαρδή

Γεώργιος Γουλιέλμος

Eelco de Bree

Εμμανουήλ Χρυσός

Ευχαριστώ

Το Θεό που μου επέτρεψε να διαβώ αυτή την οδό…

Τους γονείς μου, Νικόλαο και Αναστασία, που σε κάθε μου πτώση με βοηθούσαν να σηκωθώ …

Και όλους εκείνους που στάθηκαν αρωγοί και συνοδοιπόροι…

Περιεχόμενα - Contents

ΠΡΟΛΟΓΟΣ

Η παρούσα διδακτορική διατριβή εκπονήθηκε στο Εργαστήριο Ανατομίας υπό την επίβλεψη και επιστημονική καθοδήγηση του κυρίου Ιωάννη Τσιαούση, Αναπληρωτή Καθηγητή Ανατομίας.

Κατά τη διάρκεια της κλινικής μου άσκησης σε προπτυχιακό επίπεδο, αναγνώρισα για πρώτη φορά την καθημερινή αγωνία και αβεβαιότητα που βιώνουν τόσο οι ογκολογικοί ασθενείς, όσο και οι ιατροί που τους παρακολουθούν. Μία αγωνία που άλλοτε αφορούσε την επιτυχή έκβαση της θεραπείας με τη συχνή φράση «Γιατρέ, θα πάει καλά η θεραπεία μου?» και άλλοτε αφορούσε την έκβαση της ίδιας της νόσου τους «Γιατρέ, θα ζήσω?». Ερωτήματα, που αν και φαινομενικά απλά, είναι τόσο δύσκολο να απαντηθούν. Έτσι, η αμηχανία, ο προβληματισμός και άλλοτε η ελπίδα ή και ο φόβος, κάλυπταν το κενό της σιωπής που επικρατούσε. Γι' αυτό και προβληματιζόμουν με ποιο τρόπο θα μπορούσα να συνεισφέρω έστω και στη μερική λύση αυτής της σιωπής. Έχοντας κατασταλάξει σχετικά με τον κλινικό μου προσανατολισμό, στην ειδικότητα της Γενικής Χειρουργικής, η απόφαση εμπλοκής μου με τη μελέτη και την αντιμετώπιση του καρκίνου του παχέος εντέρου, ήταν σχεδόν αυτονόητη. Ως εκ τούτου, σε μία προσπάθεια αρχικά να κατανοήσω και στη συνέχεια να συνεισφέρω στη βελτίωση της διαχείρισης των ασθενών με καρκίνο του παχέος εντέρου και του ορθού, η βιβλιογραφική αναζήτηση μου αποκάλυψε την ύπαρξη πολλών άγνωστων περιοχών που αφορούν την πρόγνωση της έκβασης μιας θεραπείας. Ένας γιατρός που, έστω και με σχετική μόνο ασφάλεια, μπορεί να προβλέψει έγκαιρα το αποτέλεσμα της θεραπείας που χορηγεί, πετυχαίνει όχι μόνο να καθοδηγήσει αποτελεσματικότερα τον ασθενή του αλλά και να υποστηρίξει το σύνολο του περιβάλλοντός του.

Στο σημείο αυτό θα ήθελα να ευχαριστήσω τον καθηγητή μου κύριο Ιωάννη Τσιαούση, Αναπληρωτή Καθηγητή Ανατομίας, που με τη διαρκή του καθοδήγηση μπόρεσα όχι μόνο να εμβαθύνω στο χώρο της έρευνας του καρκίνου του παχέος εντέρου και ορθού, αλλά πολύ περισσότερο να γνωρίσω τις αρετές ενός ακαδημαϊκού και κλινικού ιατρού. Θερμές ευχαριστίες οφείλω επίσης στους συνεπιβλέποντες καθηγητές μου κυρίους Ιωάννη Σουγκλάκο Αναπληρωτή Καθηγητή Παθολογικής Ογκολογίας και Αριστείδη Τσατσάκη Καθηγητή Τοξικολογίας και Εγκληματολογικής Χημείας της Ιατρικής Σχολής του Πανεπιστημίου Κρήτης για τη διαρκή ενθάρρυνση, συμπαράσταση, καθοδήγηση και εμπιστοσύνη που μου έδειξαν καθ' όλη τη διάρκεια της διατριβής μου.

Ευχαριστώ θερμά τους Διευθυντές των Εργαστηρίων Τοξικολογίας και Μεταφραστικής Ογκολογίας της Ιατρικής Σχολής του Πανεπιστημίου Κρήτης που παρείχαν σημαντικό μέρος της υλικοτεχνικής υποδομής που απαιτήθηκε για την ομαλή διεξαγωγή του πειραματικού σκέλους της διατριβής καθώς και το Τμήμα Παθολογικής Ογκολογίας του Πανεπιστημιακού Γενικού Νοσοκομείου Ηρακλείου για την παροχή του πειραματικού υλικού της μελέτης.

Ευχαριστώ επίσης τους Δρ. Πολυχρόνη Στιβακτάκη, Δρ. Ελένη Βακωνάκη, Δρ. Αικατερίνη Μπερδιάκη και Δρ. Αθανάσιο Αλεγκάκη καθ'ότι στάθηκαν πολύτιμοι σύμβουλοι επί του πειραματικού και αναλυτικού σκέλους της διατριβής μου.

Ευχαριστώ την Εταιρεία Μελέτης Καρκίνου του Πεπτικού Συστήματος καθώς και την εταιρεία ToxPlus (ΚΕΝΤΡΟ ΤΟΞΙΚΟΛΟΓΙΑΣ Α.Ε.) για τη διασφάλιση απαραίτητων οικονομικών πόρων, επιτρέποντας την απρόσκοπτη διεξαγωγή των πειραμάτων της παρούσας διατριβής.

Ευχαριστώ τους δότες του πειραματικού υλικού της εργασίας, υγιείς και ασθενείς, καθώς με την ευγενική και ανιδιοτελή προσφορά του αίματός τους κατέστη δυνατή η υλοποίηση της μελέτης.

Οφείλω επίσης ευχαριστίες στις κυρίες Κυριακή Κουτσουδάκη και Μαρίνα Μαυρογιάννη του τμήματος Χημειοθεραπείας και Κλινικών Μελετών της Παθολογικής Ογκολογικής Κλινικής του Πανεπιστημιακού Γενικού Νοσοκομείου Ηρακλείου αλλά και στο προσωπικό του τμήματος Ενδοσκοπήσεων της Γαστρεντερολογικής Κλινικής του ίδιου νοσοκομείου για την ουσιαστική βοήθειά τους στη συλλογή των δειγμάτων.

Τέλος, θα ήθελα να ευχαριστήσω το Διοικητικό προσωπικό της Ιατρικής Σχολής για κάθε είδους βοήθεια που μου πρόσφεραν κατά τη διάρκεια των διδακτορικών μου σπουδών στο τμήμα.

ΠΕΡΙΛΗΨΗ

Ο κολοορθικός καρκίνος (ΚΟΚ) είναι ο τρίτος πιο κοινός τύπος καρκίνου και για τα δύο φύλα και μία από τις κύριες αιτίες θανάτου σχετιζόμενο με καρκίνο παγκοσμίως. Αυτά τα χαρακτηριστικά έχουν προσελκύσει πολλές ερευνητικές ομάδες σε όλο τον κόσμο δημιουργώντας τις κατάλληλες προϋποθέσεις για την επίτευξη σημαντικής προόδου στην αποκάλυψη της υποκείμενης μοριακής βιολογίας, στον εντοπισμό νέων διαγνωστικών test και στην εισαγωγή νέων και εξατομικευμένων θεραπευτικών επιλογών. Ωστόσο, παρά τις πολυάριθμες προσπάθειες για τον εντοπισμό αποτελεσματικών προγνωστικών εργαλείων, τα αποτελέσματα δεν είναι ιδιαίτερα ενθαρρυντικά. Αυτό συμβαίνει γιατί στις περισσότερες μελέτες ο μικρός αριθμός των υπό μελέτη ασθενών δεν επιτρέπει την ασφαλή εξαγωγή συμπερασμάτων. Παράλληλα, το σχετικά υψηλό κόστος αλλά και η πολυπλοκότητα των προτεινόμενων τεχνικών καθιστά αποτρεπτική την εφαρμογή των εν λόγω εργαλείων. Ως εκ τούτου, η τρέχουσα πρακτική στη διαχείριση του ΚΟΚ εξακολουθεί να αξιολογεί την ανταπόκριση των ασθενών κατόπιν ολοκλήρωσης της θεραπείας τους. Αυτό έχει μεγάλη σημασία, καθώς οι ασθενείς που αναπτύσσουν χημειοανθεκτικότητα, θα αναγνωριστούν με μεγάλη καθυστέρηση, αφήνοντας τόσο αυτούς όσο και τα συστήματα υγείας εκτεθειμένους να αντιμετωπίσουν τις περιττές παρενέργειες και το αυξημένο κόστος των αναποτελεσματικών θεραπειών. Έτσι, προκειμένου να βελτιστοποιηθεί η κλινική πρακτική, αναδεικνύεται ως ζωτικής σημασίας η ταυτοποίηση ευαίσθητων και εύχρηστων εργαλείων που θα παρέχουν πολύτιμες πληροφορίες σχετικά με την πρόγνωση της πορείας κάθε ασθενούς. Τέτοια εργαλεία αναμένεται να ωφελήσουν άμεσα τους ασθενείς με ΚΟΚ και τους κλινικούς ιατρούς (αφού θα είναι σε θέση να διακόψουν μια αναποτελεσματική θεραπεία σε πρώιμο στάδιο) αλλά έμμεσα και το σύστημα υγείας δεδομένης της αποδοτικότερης διαχείρισης των πόρων για την θεραπεία αυτών των ασθενών. Επί πλέον πρέπει να σημειωθεί η δυνατότητα επέκτασης της αξιολόγησης της προγνωστικής αξίας των εργαλείων αυτών και σε άλλους τύπου καρκίνου. Ένας μεγάλος αριθμός δεδομένων δείχνει ότι οι μοριακοί βιοδείκτες είναι πολλά υποσχόμενοι υποψήφιοι για αυτόν τον σκοπό. Έχοντας τα ανωτέρω υπ όψιν, η παρούσα μελέτη επικεντρώθηκε στην διερεύνηση της κλινικής αξίας δύο νέων βιοδεικτών: α) της συχνότητας μικροπυρήνων (ΣΜ) σε 55 ασθενείς με μεταστατικό ΚΟΚ (μΚΟΚ) και 21 με τοπικά προχωρημένο καρκίνο του ορθού (τπΚΟ) χρησιμοποιώντας την τεχνική cytokinesis block micronucleus assay (CBMN) και β) της ενεργότητας τελομεράσης (ΕΤ) σε 23 μΚΟΚ και 5 τπΚΟ ασθενείς χρησιμοποιώντας την τεχνική της TRAP-ELISA. Αυτοί

οι βιοδείκτες επιλέχθηκαν βάσει της στενής τους σχέσης με τη χρωμοσωμική αστάθεια και τη διαταραγμένη γενετική λειτουργία, αμφότερα ουσιαστικά γνωρίσματα στην καρκινογένεση του παχέος εντέρου. Όλοι οι βιοδείκτες αξιολογήθηκαν σε λεμφοκύτταρα περιφερικού αίματος πριν, στο μέσο και στο τέλος της θεραπείας (περίπου 0, 3 και 6 μήνες) για ασθενείς με μΚΟΚ και πριν, στο τέλος της θεραπείας και μετά από χειρουργική επέμβαση για ασθενείς με τπΚΟ. Συνολικά, η ΣΜ έδειξε σημαντική προγνωστική αξία, καθώς μια μείωσή <29% μεταξύ των μεσαίων και των αρχικών μετρήσεων μπορεί να διακρίνει μεταξύ εξελισσόμενης και σταθερής / ανταποκρινόμενης νόσου με ευαισθησία 36% και ειδικότητα 87,0%. Ακόμα φάνηκε να είναι σε θέση να αναγνωρίσει την ανταπόκριση νόσου με ευαισθησία 72,7 % και ειδικότητα 59,3%. Αναφορικά δε με την ΕΤ, δεν αποδείχθηκε στατιστικά σημαντική διαφορά μεταξύ των ομάδων ανταπόκρισης νόσου σε καμία από τις μετρήσεις (προ της έναρξης της χημειοθεραπείας p = 0.256, στη μέση της χημειοθεραπείας p=0.072, στο τέλος της χημειοθεραπείας p=0.096). Ωστόσο, παρατηρήθηκε ότι στη μέση και τελευταία μέτρηση οι ασθενείς με πρόοδο νόσου είχαν αύξηση της ΕΤ ενώ η σύγκριση των υπολοίπων ομάδων με αυτή την ομάδα (πρόοδος νόσου vs σταθερή νόσος, πρόοδος νόσου vs μερική ανταπόκριση, πρόοδος νόσου vs πλήρης ανταπόκριση) ανέδειξε μια σημαντική τάση στην αύξηση της ΕΤ στους ασθενείς με πρόοδο νόσου (μέση μέτρηση p=0.072, τελική μέτρηση p=0.096). Παρ’ όλα αυτά, δεν κατέστη δυνατή η περαιτέρω στατιστική μελέτη των ασθενών με πρόοδο νόσου στις τρεις φάσεις της χημειοθεραπείας εξ’ αιτίας της απουσίας κανονικής κατανομής των τιμών της ΕΤ πιθανά λόγω του μικρού δείγματος που μελετήσαμε. Συνοπτικά, τα ευρήματα της μελέτης αυτής καταδεικνύουν την αξία της συχνότητας των μικροπυρήνων ως προγνωστικού βιοδείκτη για την παρακολούθηση της ανταπόκρισης στη θεραπεία ασθενών με ΚΟΚ, ενώ η ΕΤ πρέπει να αξιολογηθεί σε μια μεγαλύτερη ομάδα ασθενών για να τεκμηριωθεί η πιθανή μεταβολή της κατά τη διάρκεια της χημειοθεραπείας οπότε και η κλινική της σημασία.

ABSTRACT

Colorectal cancer (CRC) is the third most common type of cancer for both sexes and one of the leading causes of cancer-related mortality worldwide. These characteristics have attracted numerous research teams across the globe, creating the right conditions for significant advances on unveiling the underlying molecular biology, identifying new diagnostic tests and expanding treatment options while introducing personalized ones. However, despite numerous attempts to identify effective prognostic and predictive tools only little progress has been made. This is because in most studies the size of patient sets is rather small which prevents safe conclusions to be drawn. At the same time, the relatively high cost and complexity of the proposed techniques makes the implementation of these tools deterrent. Therefore, current practice in CRC management continues to evaluate patient response after completion of their treatment. This is of great importance, since patients who develop chemoresistance will be identified with substantial delay, leaving both them and health care systems challenging with the unnecessary side effects and increased cost of ineffective treatments. Thus, in order to optimize clinical practice, the identification of sensitive and easy-to-use tools that will provide valuable information about the prognosis of each patient is emerging as crucial. Such tools are expected to benefit patients with CRC and clinicians directly (as they will be able to discontinue ineffective treatment at an early stage) but also indirectly the health care system given the more efficient management of resources to treat these patients. In addition, it should be noted the possibility of expanding the validation of the prognostic value of these tools to other types of cancer. It is well established that molecular biomarkers can serve as promising candidates for this purpose. In view of the above, the present study focused on validating the clinical value of two new biomarkers: a) micronuclei frequency (MNf) in 55 metastatic CRC (mCRC) and 21 locally advanced rectal cancer (laRC) patients using cytokinesis block micronucleus assay (CBMN assay) and b) telomerase activity (TA) in 23 mCRC and 5 laRC patients using TRAP-ELISA. These biomarkers were chosen on the basis of their close relationship to chromosomal instability (CIN) and aberrant genetic function, both major hallmarks in colorectal carcinogenesis. All biomarkers were evaluated in peripheral blood lymphocytes (PBLs) before, in the middle and at the end of treatment (approximately 0, 3 and 6 months) for mCRC patients and before, at the end of treatment and after surgery for patients with laRC. Overall, MNf demonstrated significant prognostic value, since a reduction of <29% between middle and initial MNf measurements can discriminate between progressive and stable/responsive disease with a sensitivity of 36% and a specificity of 87.0%. It also appeared to be able to identify responsive disease with sensitivity of 72.7% and specificity 59.3%. Regarding TA, no statistically significant difference was found between the disease response groups in any of the sampling points (before the beginning of chemotherapy $p = 0.256$, in the middle of the chemotherapy $p = 0.072$, at the end of the chemotherapy $p = 0.096$). However, it was observed that in the middle and last sampling points, the patients with progressive disease had an increase in TA while the concomitant comparison between groups (disease progression vs stable disease, disease progression vs partial response, disease progression vs complete response) revealed a significant trend to increase TA in patients with disease progression (middle sampling point $p = 0.072$, final sampling point $p = 0.096$). However, it was not possible to further statistically compare TA

values of patients with progressive disease between the three sampling points due to the absence of a normal distribution of TA values, possibly due to the small sample we studied. In conclusion, the findings of this study demonstrate the value of MN frequency as a promising prognostic biomarker for monitoring the response to treatment of patients with CRC, while TA should be evaluated in a larger group of patients to document its possible change during chemotherapy and its related clinical significance.

2. Introduction

Colorectal cancer is the third most common cancer diagnosed worldwide while at the same time it is considered as one of the leading causes of cancer-related morbidity and mortality worldwide for both sexes (1). Overall, the lifetime risk of developing colorectal cancer is about 1 in 23 (4.3%) for men and 1 in 25 (4.0%) for women. It was estimated that in 2020 147,950 individuals would be diagnosed with CRC in the United States (70.7% would suffer from colon and 29.3% from rectal cancer) while 53,200 patients would die from the disease (2). 2021's projection has not improved since it is estimated that 149,500 individuals will be affected and 52,980 will die by it (3). Unfortunately, even though the majority of patients at the time of diagnosis present with local or locally advanced (regional) disease (stages I, II and III), some 22% will present with distant metastases (stage IV disease) (4). Thankfully, owing to the great advances in CRC management and treatment, stages I-III exhibit the most favorable prognosis with overall 5-year survival rates reaching 94.7%, 88.4% and 74.3% for stages I, II and III accordingly. In addition, five-year disease-free survival rates are 91.0%, 79.8% and 63.3% in stage I, II and III accordingly. On the contrary stage IV disease has the worst survival rates since the overall five-year survival rate and the five-year disease-free survival rate are 31.5% and 18.9% accordingly (5). Apart from the impaired survival rates in stage IV disease, a significant number CRC patients with distant metastases develops resistance to their therapy at some point of the course of their treatment. Unfortunately, diagnosis of chemoresistance is most often delayed, allowing for cancer progression to take place before these patients receive second or third line treatments. At the same time, healthcare systems are dealing with an immense financial burden as a result of these treatments. Therefore, identification of accurate, cost efficient and easy-to-use tools that will be able to provide valuable prognostic and predictive information is considered to be crucial. Indeed, numerous studies have attempted to address this issue and most of them indicate molecular biomarkers as promising candidates for this purpose (6). In regards to CRC prognosis, research on current evidence related with chromosomal instability (CIN) and aberrant genetic function; both major hallmarks in colorectal carcinogenesis (7), led this doctoral thesis to investigate the clinical and possible prognostic value of two novel biomarkers (MNf, and TA) for laCRC and mCRC.

1.1 CRC Risk Factors

In order to better understand CRC etiology, several epidemiological studies have successfully identified many anthropometric, dietary, lifestyle, and pharmacological factors that influence the relative risk of CRC carcinogenesis. These factors are collectively described as risk factors. A risk factor is anything that increases a

person's chance of developing a disease and therefore, a cancer such as CRC. Although risk factors often influence the development of cancer, most of them do not directly cause cancer. However, coexistence of a number of risk factors along with an underlying genetic susceptibility increases the risk of CRC. Risk factors can be roughly divided in two groups; modifiable and non-modifiable. Modifiable, are those factors that can be changed upon the willing of the person, while non-modifiable are those that cannot be changed. Those factors that are considered to be modifiable factors for CRC development are: lack of regular physical activity, a diet low in fruit and vegetables, a low-fiber and high-fat diet, or a diet high in processed meats and refined carbohydrates, being overweight or obese, insulin resistance and subsequent hyperinsulinemia (induced by excess energy intake), increased alcohol consumption and smoking (8). On the other hand, those factors that are considered to be non-modifiable are: older age, black race, male sex, family history of CRC and personal history of CRC, hereditary conditions such as Lynch syndrome, a personal history of inflammatory bowel disease, ovarian or uterine cancer (9). On the contrary, a number of factors are identified to reduce the relative risk of CRC development (preventive factors). Such factors include increased physical activity, use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs), use of postmenopausal hormone (PMH) in women, and regular consumption of fruits, vegetables, calcium, folate, and fiber (9). Moreover, migration studies also demonstrate a higher lifetime incidence of colorectal cancer among immigrants to high-incidence, industrialized countries compared to residents remaining in their native, low-incidence countries (10). Unfortunately, it is uncertain whether changing modifiable risk factors has equal effect among those with high versus low non-modifiable CRC risk profiles. For example, Maas et al studying a breast cancer consortium of prospective studies, demonstrated that improvement in estimating absolute risk of breast cancer can identify subsets of the population at an elevated risk who would benefit most from risk-reduction strategies such as altering modifiable factors, suggesting the utility of comprehensive risk modelling (11). Taken together, these data highlight the importance of environmental influences on colorectal carcinogenesis and the need for successive implementation of primary prevention strategies by all health care systems.

1.2 CRC development

In most cases, CRC development is a long lasting that most probably will not give any symptoms until it reaches a considerable size of several centimeters. Such symptoms may be partial or complete obstruction of the affected part of the colon (leading to cramping, pain, constipation or ileus) or in some other cases bleeding, that can present as visible bleeding with bowel movements. Until then, a multistep process takes place involving a series of histological, morphological, and genetic changes that accumulate over time (12). For the most part, the natural history of colorectal cancer has been examined by comparing sequential morphological changes in patients who were able to be followed up clinically using radiography or endoscopy. According to these findings, we know that CRC typically develops from focal changes within benign, precancerous polyps (Figure 1).

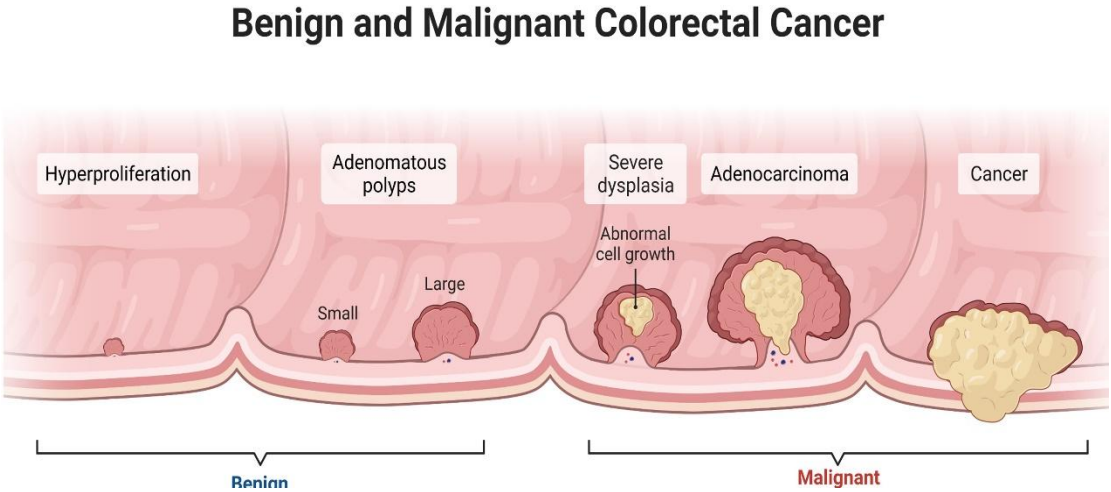

Figure 1. Illustration of the developmental timeline in colorectal carcinogenesis form benign to malignant lesions. Created with BioRender.com

These polyps are localized growths or aggregations of abnormal cells within the intestinal mucosa that protrude into the intestinal lumen (13). Polyps can develop along the entire length of the colon and rectum; however, polyps that develop within the proximal (right) side of the colon, which includes the cecum through the transverse colon up to and including the splenic flexure, account for 42% of all CRCs in the US (14). Overall, polyps can be categorized in two large groups: a) non-neoplastic polyps, including hyperplastic polyps, inflammatory polyps and hamartomatous polyps and b) neoplastic polyps, including adenomas (tubular and tubulovillous) and sessile serrated polyps (SSPs). Polyps can be sessile or pedunculated. The dividing cells in these polyps may accumulate sufficient genetic alterations by which they acquire the ability to invade the bowel wall, which is the hallmark of CRC. Over time, neoplastic polyps may acquire the ability to spread to local lymph nodes and finally to other organs (distant metastases). Fortunately, only a fraction of polyps reaches the threshold of malignancy. In general, most adenomas have a tubular histology with small, roundish, atypical glands but often develop areas of long filamentous architecture as they grow, which is described on pathology reports as villous or tubulovillous. By definition, adenomas are characterized by dysplasia (low degree of cellular and structural atypia). Tubulovillous and villous adenomas, especially those with ≥25% villous content, are typically larger in size and have a greater potential for harboring cancerous cells. In contrast, SSPs are flat and carpet like, with serrated or saw-toothed glands. SSPs include sessile serrated adenomas, traditional serrated adenomas, and mixed polyps, which have all been associated with CRC development (15,16). Even though, a polyps' size is not sufficient to presume its malignant potential, it is largely accepted that the larger a polyp is, the greater the risk of cancer. Although only 10% of even the most advanced adenomas (adenomas ≥1 cm in size or that have ≥25% villous component or high-grade dysplasia of any size) become cancerous, 60%–70% of CRCs develop from adenomas. The remaining 25%–35% of CRCs develop from SSPs (16,17). As the cells within the polyp proliferate, genetic mutations and epigenetic changes begin to accumulate. From a pathology point of view this is reflected by cytologic and histologic dysplasia (18).

1.3 Genetic alterations during CRC development

Both genetic and epigenetic alterations are common in CRC and are crucial steps for carcinogenesis. In 1990, Fearon and Vogelstein elucidated specific pathways essential to the development of CRC, consisting of accumulated mutations in multiple genes that regulate cell growth and differentiation (19). As a result, the adenoma-carcinoma sequence was proposed as the governing mechanism for normal colorectal epithelium to transform to an adenoma, an in situ carcinoma and ultimately to an invasive and metastatic carcinoma. According to it, initial genetic changes start in an early adenoma and accumulate as it transforms to carcinoma (Figure 2).

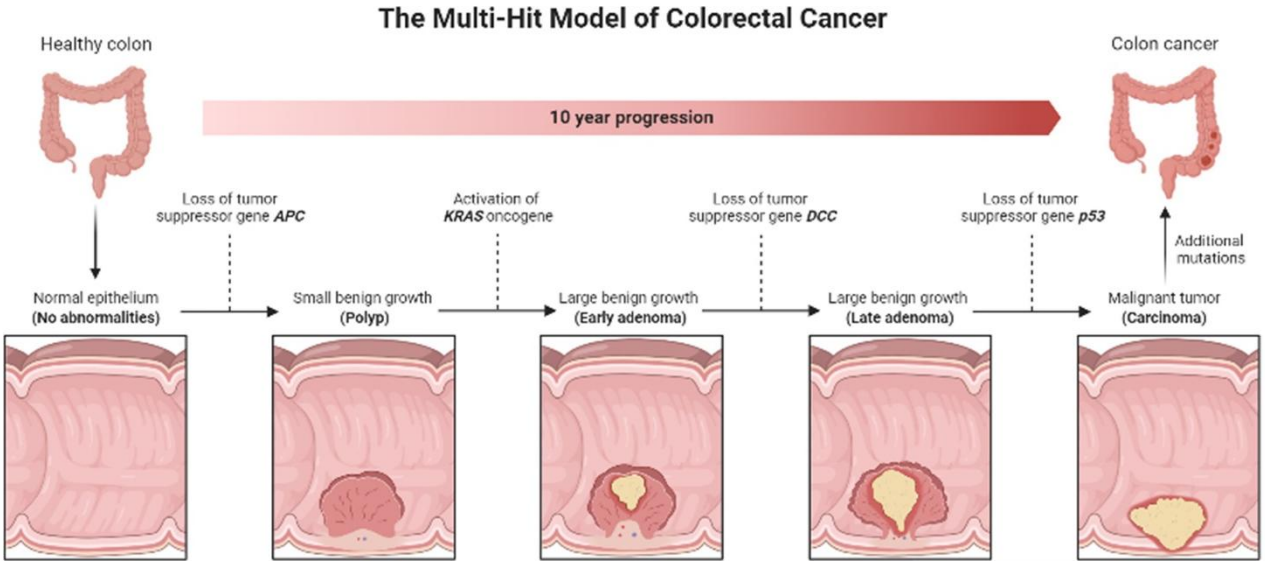

Figure 2. Graphic presentation of the multi-hit model in colorectal carcinogenesis where the primary lesion begins with a mutation in the APC gene. As other tumor suppressor genes mutate, a larger tumor will grow and eventually become malignant. Created with BioRender.com

DNA mutations can be acquired or inherited. True inherited mutations associated with CRC, such as the MLH1, MSH2, PMS2, and the APC gene mutations, are uncommon and account for approximately 5% of all CRCs. However, studying these inherited mutations, in addition to sporadically occurring APC and DNA mismatch repair mutations, has provided key insights into the stepwise genetic progression from premalignant polyps to cancer (20). There are two main genetic pathways implicated in CRC development; chromosomal instability pathway (CIN) (accounting for 70-80% of all CRCs) and microsatellite instability pathway (MSI) (5–20% of all CRCs). However, apart from these two distinct pathways of genomic instability, a third one has also been recognized; CpG Island Methylator Phenotype (CIMP) (~15 % of all CRCs) (6,21). CIN main characteristic is the extensive abnormality in chromosome number (aneuploidy) and loss of heterozygosity. CIN can be observed in several forms, including chromosomal numerical abnormalities, small sequence modifications such as base deletions or insertions, chromosomal rearrangements and gene amplification. Such alterations may include activation of proto-oncogenes (K-Ras) and inactivation of at least three tumor suppression genes, namely, loss of APC (chromosome region 5q21), loss of p53 (chromosome region 17p13), and loss of heterozygosity for the long arm of chromosome 18 (18q LOH). Recently mutations

involving other genes have been described, such as the TGFBR and PIK3CA, that are required for the adenoma-carcinoma sequence model (18). Microsatellites (MSs) are short tandem-repeated base pairs of 1–6 scattered all over the genome. For the normal human genome the number of MSs is approximately half a million. Genome studies revealed that MSs are prone to duplication errors. However these errors are usually corrected by the miss match repair (MMR) system (22,23). Consequently it is logical to assume that a defective MMR system would result in the accumulation of DNA mistakes and thus MSI. Indeed, MSI arises by the inhibition of MMR system either via defective methylation of MLH1 in CpG island or point mutation of any MMR genes (hMLH1, hMSH2, hMSH6, PMS1 and PMS2) especially hypermethylation of hMLH1 promoter (6). It is estimated that about 15–20% of CRC patients present MSI with a small fraction of which 2–4% are related to HNPCC (24). In order to estimate MS status, Bethesda panel was agreed in which five MS loci were included (BAT25, BAT26, D5S346, D2S123, and D17S250) (25). However, some researchers suggested an expanded Bethesda panel include 10 loci. Based on this panel, MSI can be divided into three groups: MSI-high (MSI-H), defined as having ≥30% unstable loci using mononucleotide or dinucleotide markers (26); MSI-low (MSI-L), with 10–30% unstable loci; and microsatellite stable (MSS), with <10% unstable loci (26). MSI status varies according to a given CRC stage: Stage II CRC exhibits high prevalence of MSI (20%) while in stage IV CRC MSI is approximately 4% (27,28). Moreover, differences based on the MSI status are found when prognosis is examined. For example, cases with MSI-H CRC share a better prognosis than that with MSS CRC (6). CpG island accounts for >70% of CG sequences that extend to 0.4 kB on the genome (29). Even though hypermethylation of CpG island cytosine represents a hallmark for cancer progression, both hypomethylation and hypermethylation may lead to the transformation of normal mucosa to adenoma and subsequently to the development of CRC (30). Disturbance of epigenetic programming (epigenetic modification including DNA methylation, histone modification and post-transcriptional gene regulation) is closely related to the development of CRC (31). It is reported that a wide spectrum of aberrant methylated genes in CRC, regulates crucial functions in the normal cell regarding proliferation and maintenance of genome stability (32). These genes include WiF-1, AIX4, PGR, FBNI, P53, TIMP3, SEPT9, MGMT, Vimentin, GATA4 CNRIP1, FBN1, INA, MAL, SNCA and SPG20 (31–33). It should be noted that APC is the most common initial gene mutated in familial/inherited and sporadic colon cancer (approximately 90% of CRC caces). APC gene regulates the Wnt signaling pathway via encoding a multifunctional protein. Specifically, APC regulates Wnt pathway through the destruction of the transcription factor β-catenin, which enhances the activity of Wnt pathway. Hence, APC conversely organizes Wnt signaling (34). In addition, APC gene is involved in cell cycle regulation, cytoskeleton stabilization, intracellular adhesion, as well as apoptosis. In an attempt to determine the exact role of APC gene in CRC, Dow et al (35) investigated whether APC mutation is essential for CRC protection. For their study a CRC mouse model with inhibited APC was used. According to their findings, inhibiting APC gives rise to adenomas in colon and small intestine (35). Liang et al conducted a meta-analysis study to correlate APC polymorphism (D1822V, E1317Q, I1307K) and CRC risk. They concluded that E1317Q significantly increased adenoma risk. However, I1307K is associated with high risk of CRC (36). CRC-related tumor suppressor genes are thought to be altered in the early phase of cancer development, and APC mutation is the first step in the translation of normal mucosa to neoplastic

tissue, leading to the activation of the WNT pathway. Subsequent mutations that occur in genes, such as KRAS, TP53, SMAD4 and type II TGF-β receptor (TGFBR2), lead to the progression from polyp to cancer similar to the process that takes place in other gastrointestinal carcinomas (37). Interestingly, it is observed that the two main types of neoplastic polyps (adenomas and SSPs) are generally characterized by distinct genetic alterations. The CIN pathway, generally associated with traditional adenomas, is observed in 65%–70% of all sporadic cancers and is characterized by a cascade of accumulating mutations. Typically, the first mutations that develop are within the APC gene, which affects chromosome segregation during cell division. Subsequent mutations then develop in the KRAS oncogene, which has downstream effects on cell growth, differentiation, motility, and survival. Over time, these mutations can cause a loss of function of the p53 gene, which is a master regulator of transcription and apoptosis, thus impacting a wide range of cellular functions that ultimately results in carcinogenesis (38). In contrast, development of SSPs tends to begin with mutations in the BRAF gene, which results in altered growth signaling and loss of apoptosis (16). KRAS mutations can also occur in SSPs, but they are much less frequently associated with SSPs than adenomatous polyps (39). Figure 3 illustrates the various gene mutations identified in colon and rectum according to the site of the primary lesion.

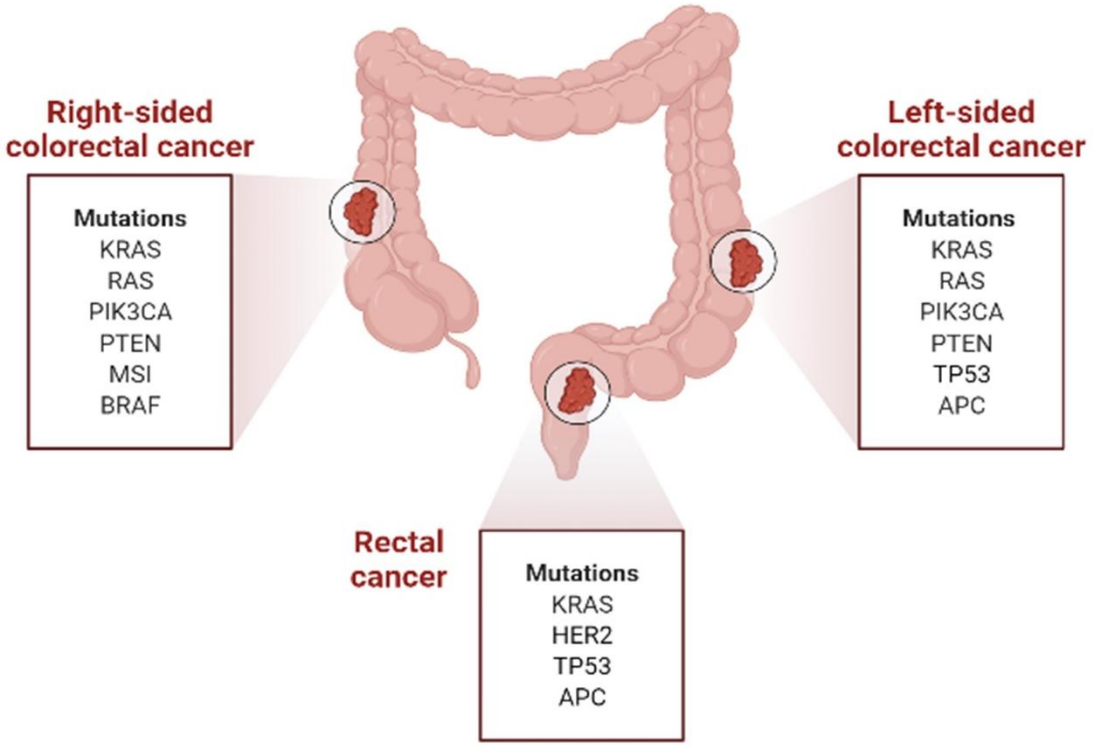

Figure 3. Presentation of the variability in gene mutations of CRC according to the site of the primary lesion. Created with BioRender.com

Another common epigenetic alteration seen in serrated lesion-based CRC is aberrant gene promoter region hypermethylation. Promoter region methylation inhibits gene transcription, functionally turning affected genes “off ”. This gene deactivation impacts many genes including those regulating other growth-promoting genes(39). Aberrantly methylated genes associated with CpG island methylator phenotype include, among others, the bone morphogenic protein 3 (BMP3)25 and N-Myc downstream-regulated gene 4 (NDRG4) (40,41). MSI can occur in both adenomatous

and serrated polyps and is associated with germline mutations in DNA mismatch repair genes (eg, in hereditary nonpolyposis colorectal cancer) as well as sporadic mutations due to aberrant methylation of the MLH1 promoter regions (associated with CpG island methylator phenotype).

1.4 Imaging modalities for CRC staging

Treatment strategy for colon cancer is guided by adequate staging. Complete colonoscopy and multi-detector computed tomography (MDCT) scan of the chest, abdomen, and pelvis should be performed. MDCT remains the main imaging modality for preoperative planning, metastatic liver lesion detection and tumor surveillance. MDCT accuracy rate for assessing lower stage lesions is not as good as that for advanced lesions. This discrepancy relates to the limited ability of MDCT to determine depth of bowel wall penetration. However, abdominal/pelvic MDCT has a high negative predictive value. The specificity for detecting lymph nodes involved with tumor is approximately 50%. In addition, the modality offers the ability of a rapid global evaluation and demonstration of complications (perforation, obstruction, etc.) that may not be clinically apparent (42). Magnetic resonance imaging (MRI) has equal accuracy to MDCT for local staging of colonic neoplasms. Accuracy in identification of lymph node metastases is also equal to MDCT, and slightly superior for detection of liver metastases. MRI may be beneficial in determining involvement of the adjacent organs. MRI may also be considered in preoperative evaluation of patients with sensitivity to iodinated contrast material, particularly in the evaluation of the liver (43). MRI and contrast-enhanced ultrasonography (US) should be considered as problem solving techniques for characterization of indeterminate liver lesions (42). Computed tomographic colonography (CTC) can accurately identify all colorectal masses but may overcall stool as masses in poorly distended or poorly prepared colons. CTC has an overall staging accuracy of 81% for colorectal cancer and is superior to barium enema in visualizing colonic segments proximal to obstructing colorectal lesions. Furthermore, the method can identify synchronous lesions in patients with colorectal masses, and image the proximal colon in patients with obstructing colorectal lesions (44). FDG-PET is not recommended for initial staging. It could be used in patients at high surgical risk when there is a strong probability of metastatic disease invisible on CT or MRI. However, the role of FDG PET/CT is not yet clear owing to the small number of studies (45). Also, brain and bone scintigraphic scans are only indicated in patients with relevant symptoms. Rectal cancers are categorized according to their distal edge measured from the anal verge, by rigid or flexible endoscopy, accompanied by biopsy, and MRI. It is important to note that according to the current clinical practice RCs require an initial (diagnostic staging) and a pre-operative staging. For the initial staging, rigid endoscopy and MRI are more reliable in detecting the exact location and the size of the tumor. By rigid proctoscopy, rectal cancer is categorized as: low (up to 5 cm), middle (from >5 to 10 cm) or high (from >10 up to 15 cm). The accurate diagnosis of tumor localization and local extension (T stage), lymph node involvement (N-stage), extramural vein status and potential CRM positivity is essential for defining the treatment strategy. Subclassification of T1 cancers is based upon depth of invasion into the submucosal layer: sm1 upper third, sm2 middle third and sm3 lower third. Alternatively the

millimetric depth of submucosal invasion could be used, where an invaded depth of more than 1 mm is an important predictor for possible lymph node involvement (46,47). Endorectal ultrasound (ERUS) and endorectal MRI have similar accuracy in the differentiation between T1 sm1/sm2 and sm3 and furthermore between superficial (T1 and/or T2) and T3 tumors (48). MRI with use of an endorectal coil offers the maximum amount of information by a single modality in the staging of rectal cancer (49). However, endorectal imaging is not an adequate method for the assessment of local tumor extent in bulky T3 or T4 tumors. Likewise, ERUS or MRI can measure sphincter infiltration with comparable accuracy. Although ERUS is accurate in assessing early-stage low tumors (T1 and T2), with a sensitivity of 94% and specificity of 86% (50), it performs inadequately for advanced rectal tumors, leading to substantial preoperative overstaging and consequent overtreatment, because differentiation between peritumoral inflammation or fibrosis and true tumor is not possible (51,52) and the inability of the method to assess CRM or to identify lymph nodes close to the mesorectal fascia, but also to depict extramural vascular invasion (EMVI). Thin-section MRI with 3-mm slices and a small field of view is now used to identify several prognostic features that will allow better selection of patients who will benefit from more intensive treatment (53). MRI or multidetector-row CT (MDCT) have an equal accuracy in distinguishing T3 from T4 tumors in the middle and higher rectum (54). However, MDCT does not correlate well enough with MRI findings to replace it in rectal cancer staging (55). The main limitation of T staging is that T3 tumors are the majority of rectal cancers seen at presentation which, however, comprise a very heterogeneous group regarding local recurrence and survival rates. From existing pathological studies, it is clear that patients with more than 5 mm of extramural spread should be identified because they have a markedly worse prognosis than those with T3 tumors <5 mm of spread (56). Thus, the distinction between T2 stage and T3 stage is not relevant, when the T3 tumor presents a less than 2 mm spread (57). In summary, MRI has been shown to accurately identify the depth of extramural invasion, the presence of lymph node metastases, EMVI and CRM involvement. By demonstration of accurate measurement of the depth of extramural tumor spread, the MERCURY Study enabled accurate preoperative prognosis (58). Given the fact that major pelvic surgery for locally advanced rectal cancer is associated with postoperative morbidity of 40-50% (59), selecting patients for observation (ypT0, ypN0) or for local excision (ypT0-2, ypN0) after chemoradio therapy (CRT), although within research protocols at present, represents a major challenge. Hence accurate restaging is of paramount importance. Most of the studies suggest that none of the available imaging modalities (ERUS, standard MRI, CT, or FDG-PET) are sufficiently accurate in identifying complete remission after CRT, offering positive predictive value (PPV) as low as 17-50% (60,61). Downsizing of rectal cancer after CRT to ypT0-2 tumor can be predicted accurately by using MRI, with a high PPV at the cost of a lower NPV, because of diffuse fibrosis usually seen after RT therapy and inability to distinguish between only fibrosis and fibrosis with tumor cell nests. After volumetric analysis, when the initial tumor volume is less than 50 cm3 and the decrease in volume after CRT is more than 75%, then a ypT0-2 can be predicted (57). It has recently been shown that MRI can identify the presence of residual tumor foci with good agreement between MRI tumor regression grade and histopathologic tumor regression grade (62). Optimally, pre and post CRT MRI scans should be done with the same, optimized high-resolution MRI protocol using the same parameters, allowing for a more accurate assessment of tumor regression,

potential operability and type of surgery to be offered. Parameters to be reassessed are in particular: i) tumor height and reduction in craniocaudal length, which may have an impact on the choice of operation; and ii) new CRM status, clear of areas of fibrosis, which represents the margins of resection, rather than tumor regression, which may still harbor malignant cells (63). The morphological criteria (i.e. signal heterogeneity, irregular borders and size) used in pre-treatment MRI for evaluation of nodal status still apply after CRT. Accurate non-invasive MRI assessment of regression of poor-prognosis stage N2 disease to N0 or N1 indicates effective therapy (64). The most recent multicenter prospective study in the field (MERCURY trial) (65) evaluated the prognostic relevance of post-neo-adjuvant therapy MRI assessment of tumor stage, nodal status, CRM, and MRI assessment of tumor regression grade (mrTRG) system in association with overall survival, disease-free survival and local recurrence in patients undergoing neo-adjuvant therapy and TME surgery. The study showed a significant correlation between radiologically determined tumor response and long-term outcomes and has shown that MRI assessment of tumor regression grade after preoperative therapy predicts overall survival, disease-free survival and patient prognosis, before surgery. Therefore, high-resolution MRI protocols with assessment of post-treatment TRG and CRM status can effectively help the MDT individualize treatment options before definitive surgery. Diffusion-weighted MRI (DWI) helps differentiate between residual tumor, which possesses a higher cellularity and shows a high signal and fibrosis [62]. By combining morphological with functional imaging information, MRI and DWI can significantly improve sensitivity for selection of complete responders and thus reduce interpretation difficulties when the primary tumor bed has become fibrotic after RT treatment, resulting in less overestimation of tumor in patients with a complete tumor response. Nevertheless, interpretation errors can still occur with DWI (66). Also, adding DWI to T2-weighted imaging can improve the prediction of tumor clearance in the mesorectal fascia after neo-adjuvant CRT before curative surgery, compared with T2-weighted imaging alone in patients with locally advanced rectal cancer. However, the challenge of small tumor cell-clusters identification, difficult to detect even at histology, still remains beyond the detection level of any imaging modality. Although PET using 18FDG tracer can be of some help in the evaluation and prediction of response to CRT, PET is less reliable in identifying complete responders after completion of CRT and cannot differentiate between ypT0-2 and ypT3-4 tumors or fibrosis with or without tumor (67). PET is reserved for the evaluation metastatic or recurrent disease, but its role for assessing mesorectal nodes is limited because mesorectal nodes are most frequently found at the level of the tumor and the avid metabolic uptake of 18FDG tracer within the primary tumor obscures visualization of the nodes (68).

1.5 CRC Staging

Treatment decisions can be made with reference to the TNM (tumor, node, metastasis) classification which is used for both clinical and pathologic staging (Table 1). It is recommended that at least 12 lymph nodes be examined in patients with colon and rectal cancer to confirm the absence of nodal involvement by tumor (69–71). This recommendation takes into consideration that the number of lymph nodes examined is a reflection of the aggressiveness of lymphovascular mesenteric dissection at the time of surgical resection and the pathologic identification of nodes in the specimen.

Retrospective studies demonstrated that the number of lymph nodes examined in colon and rectal surgery may be associated with patient outcome (72,73). Rectal cancers (RC) however, constitute a rather particular subset of CRCs. This is because it is observed that if same rules for colon cancer treatment are applied for rectal cancers, the outcome will be suboptimal (74). Therefore, it is recommended that RCs are divided into four groups: very early (some cT1), early (cT1–2, some cT3), more advanced (cT3, some cT4) and locally advanced (cT4). Factors other than clinical T-stage, such as tumor height, proximity to the circumferential resection margin (CRM), cN-stage, and vascular and nerve invasion are also relevant. The terms 'favorable or early or good', 'intermediate or bad' and 'locally advanced or ugly' can also be applied to categorize rectal cancers. Currently, in clinical practice, the term 'locally advanced' has been commonly used for the 'intermediate/bad' group, but is best reserved for the truly 'locally advanced/ugly' tumors. Accurate clinical staging is required to determine the need for neo-adjuvant therapy or an enhanced surgical procedure. Because oncological outcomes strongly depend on accurate diagnosis, staging and pursuing the optimal therapeutic strategies, patients with rectal cancer should be treated in specialized centers with high volume of referred cases and by an MDT which involves surgeons, histopathologists, radiologists, medical and RT oncologists (57,75,76). An optimum therapeutic strategy and adequately executed surgery aims to lower morbidity and mortality, local recurrence rates below 10%, and overall survival above 70%. Structural surgical training and quality assurance are also prerequisites for the continuous improvement of outcomes (77,78). In case of a rectal cancer, the gross description of the histology report must include the length of surgical specimen, the location of the tumor (at or below the peritoneal reflection, or the distance from the dentate line if an abdominoperineal excision is performed, the tumor size (3 dimensions), the length of proximal and distal margins, the depth of invasion, tumor perforation, other lesions not related with the tumor such as Crohn's disease, ulcerative colitis, polyp, familial adenomatous polyposis, and the number of lymph nodes retrieved. Blocks should be taken from the area closest to the CRM and any area where the tumor extends to within less than 3 mm from the margin (79,80). Standard microscopic description must include: i) histologic type, according to WHO classification. Mucinous component, presence of signet ring carcinoma (>50% signet ring), and medullary carcinomas should be mentioned, as these elements affect prognosis (81) ii) histologic grade (low grade: >50% glandular formation; high grade: <50% glandular formation) iii) T status. In pT1 lesions, distance of tumor from the resection margin, vascular or lymphatic invasion and the depth of invasion into submucosa must be reported iv) total number and number of involved lymph nodes (≥12 lymph nodes must be found to predict actual lymph node status). Any node containing malignant cells, irrespective of size and even with smooth contour is considered as involved. Extramural tumor nodules measured >3 mm without evidence of residual lymph node tissue are considered as positive lymph nodes v) blood, lymphatic vessel, tumor budding, and perineural invasion (each one an independent prognostic factor) vi) presence of tumor infiltrating lymphocytes; and vii) surgical margin status and residual tumor classification [R classification system with four different grades: Rx (presence of residual tumor cannot be assessed), R0 (no residual tumor - distance from the closest margin must be reported), R1 (microscopic residual tumor) and R2 (macroscopic residual tumor)] (81). In addition, the microscopic description must include: i) TME status: Mesorectal defects are classified into three categories: a) complete: mesorectum is intact, smooth with only minor irregularities

without defect greater than 5 mm; b) moderate: moderate bulk of mesorectum but irregularity of the mesorectal surface. Muscularis propria is not visible with the exception of the area of insertion of levator muscles; and c) incomplete: little bulk of mesorectum with defects down into muscularis propria. There is also a grading system used to determine completeness of the mesorectal excision: grade 1 indicates incomplete resection; grade 2 nearly complete; and grade 3 complete resection (79), ii) circumferential resection margin (CRM) status: CRM may be infiltrated either by direct spread or incomplete removal of Iymph nodes that are situated just under the mesorectal fascia. There is an increased risk for local recurrence, distant metastases, and poorer survival, when the CRM is involved, or measures less than 1 mm, or surgically violated to the level of tumor deposits, and iii) tumor regression after preoperative treatment (TRG): TRG is determined by the amount of residual viable tumor versus the fibrous or fibro-inflammatory tissue within the gross tumor mass. One of the methods is that of the Dworak scoring with five grades: grade 0: no regression; grade 1: minimal regression with obvious fibrosis; grade 2: moderate dominantly fibrotic changes with few tumor cells or groups of cells; and grade 4: total regression (82). In case of total regression, the pathologist is advised to slice and block the whole fibrotic area. In some cases, the only finding is the presence of acellular mucin pools within the tumor gross mass and this must be regarded as no residual tumor (83).

Stage	**TNM**	**Description**
0	Tis, N0, M0	Tis = Carcinoma in situ, intramucosal carcinoma (involvement of lamina propria with no extension through muscularis mucosae)
		N0 = No regional lymph node metastasis
		M0 = no evidence of tumor in distant sites or organs (This category is not assigned by pathologists)
I	T1, T2, N0, M0	T1 = Tumor invades the submucosa (through the muscularis mucosa but not into the muscularis propria)
		T2 = Tumor invades the muscularis propria
		N0 (see above)
		M0 (see above)
IIA	T3, N0, M0	T3 = Tumor invades through the muscularis propria into pericolorectal tissues
		N0 (see above)
		M0 (see above)
IIB .	T4a, N0, M0	T4a = Tumor invades through the visceral peritoneum (including gross perforation of the bowel through tumor and continuous invasion of tumor through areas of inflammation to the surface of the visceral peritoneum)
		N0 (see above)
		M0 (see above)
IIC	T4b, N0, M0	T4b = Tumor directly invades or adheres to adjacent organs or structures
		N0 (see above)
		M0 (see above)
IIIA	T1, N2a, M0	T1 (see above)

		N2a = Four to six regional lymph nodes are positive.
		M0 (see above)
	T1–2, N1/N1c, M0	T1 (see above)
		T2 (see above)
		N1 = One to three regional lymph nodes are positive (tumor in lymph nodes measuring ≥0.2 mm), or any number of tumor deposits are present and all identifiable lymph nodes are negative
		N1c = No regional lymph nodes are positive, but there are tumor deposits in the subserosa, mesentery, or nonperitonealized pericolic, or perirectal/mesorectal tissues
		M0 (see above)
IIIB	T1–T2,N2b, M0	T1 (see above)
		T2 (see above)
		N2b= Seven or more regional lymph nodes are positive
		M0 (see above)
	T2–T3, N2a, M0	T2 (see above)
		T3 (see above)
		N2a (see above)
		M0 (see above)
	T3–T4a, N1/N1c, M0	T3 (see above)
		T4a (see above)
		N1 (see above)
		N1c (see above)
		Mo (see above)
IIIC	T3–T4a, N2b, M0	T3(see above)
		T4 (see above)
		T4a (see above)
		N2b (see above)
		M0 (see above)
	T4a, N2a, M0	T4a (see above)
		N2a (see above)
		M0 (see above)
	T4b, N1–N2, M0	T4b = Tumor directly invades or adheres to adjacent organs or structures
		N1 (see above)
		N1a (see above)
		N1b (see above)
		N1c (see above)
		N2 (see above)
		N2a (see above)
		N2b(see above)
		M0 (see above)
IVA	Any T, Any N, M1a	M1a = Metastasis to one site or organ is identified without peritoneal metastasis
IVB	Any T, Any N, M1b	M1b = Metastasis to two or more sites or organs is identified without peritoneal metastasis
IVC	Any T, Any N, M1c	M1c = Metastasis to the peritoneal surface is identified alone or with other site or organ metastases

Table 1. TNM classification for CRC staging according to AJCC: Colon and rectum. In: Amin MB, Edge SB, Greene FL, et al., eds.: AJCC Cancer Staging Manual. 8th

ed. New York, NY: Springer, 2017, pp 251–74. T = primary tumor; N = regional lymph nodes; M = distant metastasis.

1.6 CRC treatment strategies

Typically, the ideal CRC treatment is to achieve complete removal of the primary tumor and elimination of any circulating cancer cells and metastases. To achieve this, surgical intervention is considered to be the cornerstone with the addition of pharmaceutical intervention (chemotherapy, targeted therapy) when needed (84). However, as stated above nearly a quarter of CRCs are diagnosed at an advanced stage with metastases, and 20% of the remaining cases may develop metachronous metastases, which result in difficulties in curative surgical control and subsequent tumor-related deaths (85,86). For those patients with unresectable lesions or who are intolerant to surgery, the goal is maximum shrinkage of the tumor and suppression of further tumor spread and growth. In this case, radiotherapy and chemotherapy are the leading strategies for controlling disease in such patients. Of note, in some cases, chemotherapy or radiotherapy might be applied before or after surgery as neoadjuvant or adjuvant treatment to maximally reduce and stabilize the tumor (87–89). Treatment decisions can be made with reference to the TNM. Adjuvant therapy is a systemic treatment administered after primary tumor resection with the aim of reducing the risk of relapse and death. Every treatment option, including observation alone, needs to be discussed with patients according to their characteristics (performance status, age, comorbidities and patient preference) and to cancer features (pathological stage, grading and overall risk of relapse). Adjuvant treatment is recommended for stage III and 'high-risk' stage II patients. The first issue is therefore how to define the risk. Five-year survival after surgical resection alone is: for stage I 85%–95%, stage II 60%–80%, stage III 30%–60%. Another important problem is tailoring the decision to each individual patient. In this context, the most debated issue is the impact of patient age on decision making. The median age of patients presenting with colorectal cancer is 72 years whereas the median age of patients in clinical trials is 63 years, and <10% of patients above age 70 are accrued in the studies. When facing an elderly patient (>70) with a resected high-risk colorectal cancer one must keep in mind that: (i) the life expectancy of a 70-year-old otherwise healthy individual is ~8 years for men and 14 years for women; (ii) toxicity of chemotherapy is similar below and above age 70 [II]; (iii) the efficacy of adjuvant treatments is similar in elderly people to that in the general population [II]; (iv) recent data from pooled analysis suggest caution in treating elderly patients with novel chemotherapy drugs (chiefly, oxaliplatin) in the adjuvant setting. Recently, nomograms have been developed and are available for resected colon cancer. These statistically based tools attempt to provide all proven prognostic factors and to quantify the risk of 5- and 10-year death as precisely as possible.

1.6.1 Stage 0 CRC Standard treatment options for stage 0 colon cancer include a) local excision or simple polypectomy with clear margins and b) colon resection for larger lesions not amenable to local excision. For early CRC stage 0 or partly stage I (T1) local excision using colonoscopy could be considered, particularly in patients

with co-morbidities. If histology shows clear margins of resection, well-differentiated tumor (G1, G2) and no lymphatic invasion, an expectant policy is recommended, as local recurrence is not very likely and lymph node metastasis may occur in only up to 4%. In case histology shows incomplete resection margins, a poorly differentiated lesion (G3, G4) or lymphatic invasion, surgical curative resection should follow, as local recurrence is very likely and lymph node metastasis may occur in up to 20% of the cases (90). Complete endoscopic polypectomy should be performed whenever the morphological structure of the polyp permits. The presence of invasive carcinoma in a polyp requires a thorough review with the pathologist for histological features that are associated with an adverse outcome. Making the decision to undergo surgical resection for a neoplastic polyp that contains invasive carcinoma involves the uncertainties of predicting and balancing adverse disease outcome against operative risk. Unfavorable histological findings include lymphatic or venous invasion, grade 3 differentiation, level 4 invasion (invades the submucosa of the bowel wall below the polyp) or involved margins of excision. Although level 4 invasion and involved margins of excision are two of the most important prognostic factors, their absence does not necessarily preclude an adverse outcome. Several staging systems to stratify the aggressiveness of polyps have been proposed, like involvement of submucosa (sm1, sm2, sm3, involves the superficial, middle and deep thirds of the submucosa, respectively), invasion into the stalk and absolute thickness of the invasive tumor beyond the muscolaris mucosae. When unfavorable histological features are present in a polyp from a patient with an average operative risk, resection is recommended. The pedunculated polyp with invasive carcinoma confined to the head with no other unfavorable factors carries minimal risk of an adverse outcome. The consensus is that endoscopic polypectomy is adequate treatment with proper follow-up examination. Invasion of the stalk but with clear margins of excision and favorable histological features may be treated with endoscopic polypectomy with a similar risk to that of level 2 invasion (invades the muscularis mucosa but is limited to the head and neck of the stalk). Pedunculated polypoid carcinomas can be treated using the same criteria as other pedunculated polyps with invasive carcinoma. Invasive carcinoma in a sessile polyp usually should be interpreted as having level 4 invasion. Consequently, standard surgical resection is recommended in patients with average operative risk.

1.6.2 Stage I colon cancer For resectable colonic carcinoma (stage I, II, III), the oncologically optimal surgical procedure is a curative (R0) colectomy with adequate proximal and distal resection bowel margins, and en-bloc complete removal of the respective to the resected segment mesocolon (Complete Mesocolic Excision - CME) with all regional lymph nodes (91).

1.6.3 Stage II CRC Apart from the surgical excision of the tumor following the principles of CME, the potential value of adjuvant chemotherapy for patients with stage II colon cancer remains controversial. The general consensus suggests that patients with stage II are at high risk if they present at least one of the following characteristics: lymph nodes sampling <12; poorly differentiated tumour; vascular or lymphatic or perineural invasion; tumour presentation with obstruction or tumour perforation and pT4 stage. Although subgroups of patients with stage II colon cancer may be at higher-than-average risk for recurrence (including those with anatomic features such as tumor adherence to adjacent structures, perforation, and complete obstruction) (92,93), evidence is inconsistent that adjuvant fluorouracil (5-FU)–based chemotherapy is associated with an improved OS compared with surgery alone.

Features in patients with stage II colon cancer that are associated with an increased risk of recurrence include: a) Inadequate lymph node sampling, b) T4 disease, c) Involvement of the visceral peritoneum and d) A poorly differentiated histology. The GRECCR-03 (NCT00046995) (94) and NCRI-QUASAR1 (NCT00005586) (95) trials evaluated the use of systemic or regional chemotherapy or biologic therapy. Investigators from the National Surgical Adjuvant Breast and Bowel Project have indicated that the reduction in risk of recurrence by adjuvant therapy in patients with stage II disease is of similar magnitude to the benefit seen in patients with stage III disease treated with adjuvant therapy, though an OS advantage has not been established (96). A meta-analysis of 1,000 stage II patients whose experience was amalgamated from a series of trials indicates a 2% advantage in disease-free survival at 5 years when adjuvant therapy–treated patients treated with 5-FU/leucovorin are compared with untreated controls (97). Based on these data, the American Society of Clinical Oncology issued a guideline stating "direct evidence from randomized controlled trials does not support the routine use of adjuvant chemotherapy for patients with stage II colon cancer.

1.6.4 Stage III CRC Stage III colon cancer denotes lymph node involvement. Treatment options include: a) Wide surgical resection and anastomosis, b) following surgery the standard treatment is a doublet schedule with oxaliplatin and 5FU/folinic acid (LV) (FOLFOX4 or FLOX). When oxaliplatin is contraindicated monotherapy with FU/LV, mostly with infusional schedules (DeGramont, AIO regimen), or oral fluoropyrimidines (capecitabine) can be employed. Studies have indicated that the number of lymph nodes involved affects prognosis; patients with one to three involved nodes have a significantly better survival than those with four or more involved nodes. Adjuvant chemotherapy options may include:

a) **Capecitabine** A multicenter European study compared capecitabine (1,250 mg/m2) administered twice daily for days 1 to 14, then given every 21 days for eight cycles against the Mayo Clinic schedule of 5-FU and low-dose LV for patients with stage III colon cancer. The study demonstrated that disease free survival at 3 years is equivalent for patients who received capecitabine or 5-FU/LV (hazard ratio [HR], 0.87; $P < .001$). Hand-foot syndrome and hyperbilirubinemia were significantly more common for patients receiving capecitabine, but diarrhea, nausea or vomiting, stomatitis, alopecia, and neutropenia were significantly less common. Of patients receiving capecitabine, 57% required a dose modification. For patients with stage III colon cancer in whom treatment with 5-FU/LV is planned, capecitabine is an equivalent alternative (provides equivalent outcome to intravenous 5-FU/LV).

b) **Oxaliplatin** In the 2,246 patients with resected stage II or stage III colon cancer in the completed Multicenter International Study of Oxaliplatin/Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer (MOSAIC [NCT00275210]) study, the toxic effects and efficacy of FOLFOX-4 (oxaliplatin/LV/5-FU) were compared with the same 5-FU/LV regimen without oxaliplatin administered for 6 months. Based on results from the MOSAIC trial, adjuvant FOLFOX-4 demonstrated prolonged OS for patients with stage III colon cancer compared with patients receiving 5-FU/LV without oxaliplatin. The preliminary results of the study with 37 months of follow-up demonstrated a significant improvement in DFS at 3 years (77.8% vs. 72.9%; $P = .01$) in favor of FOLFOX-4. When initially reported, there was no difference in overall survival (OS). Further follow-up at 6 years demonstrated that the OS for all patients

(both stage II and stage III) entered into the study was not significantly different (OS, 78.5% vs. 76.0%; HR, 0.84; 95% confidence interval [CI], 0.71–1.00). On subset analysis, the 6-year OS in patients with stage III colon cancer was 72.9% in the patients receiving FOLFOX-4 and 68.7% in the patients receiving 5-FU/LV (HR, 0.80; 95% CI, 0.65–0.97; P = .023). Patients treated with FOLFOX-4 experienced more frequent toxic effects consisting mainly of neutropenia (41% >grade 3) and reversible peripheral sensorial neuropathy (12.4% >grade 3). In a randomized phase III study (NSABP C-07 [NCT00004931]), 2,407 patients with stage II or stage III colon cancer were randomly assigned to adjuvant 5-FU/LV or fluorouracil-leucovorin-oxaliplatin (FLOX) (weekly 5-FU/LV with oxaliplatin administered on weeks 1, 3, and 5 of each 6-week cycle). DFS was the primary endpoint of the study. DFS was significantly longer in the treatment group who received FLOX, but OS was not significantly different. The DFS rate was 69.4% for patients who received FLOX and 64.2% for patients who received 5-FU/LV (HR, 0.82; 95% CI, 0.72–0.93; P = .0034). The OS rate at 5 years was 80.2% for patients who received FLOX and 78.4% for patients who received 5-FU/LV (HR, 0.88; 95% CI, 0.75–1.02; P = .08). Given the high rate of disabling neuropathy, the duration of oxaliplatin adjuvant therapy became an open question. The International Duration Evaluation of Adjuvant Therapy (IDEA) collaboration consisted of six separate randomized trials with regimens of 6 months versus 3 months of adjuvant oxaliplatin-based chemotherapy. The IDEA study was a prospective, preplanned pooled analysis of these concurrently conducted studies to evaluate the noninferiority of adjuvant therapy of either FOLFOX or CAPOX administered for 3 months versus 6 months. Noninferiority could be claimed if the upper limit of the two-sided 95% CI of the HR did not exceed 1.12. From June 2007 through December 2015, 13,025 patients with stage III colon cancer were enrolled in six concurrent phase III trials. Of these patients, 12,834 patients met the criteria for intention-to-treat analysis. At a median follow-up of 41.8 months, noninferiority of 3 months versus 6 months was not confirmed in the modified intention-to-treat population (HR, 1.07; 95% CI, 1.00–1.15, P = .11 for noninferiority of 3 months). The 3-year DFS rates were 74.6% in the 3-month group and 75.5% in the 6-month group. Neurotoxicity of grade 2 or higher was lower in the 3-month group (16.6% for patients who received FOLFOX and 14.2% for patients who received CAPOX) than in the 6-month group (47.7% for patients who received FOLFOX and 44.9% for patients who received CAPOX). Moreover, all other toxicities were substantially lower with 3 months of treatment than with 6 months. A subgroup analyses observed the following: Among patients receiving FOLFOX, 6 months of therapy was superior to 3 months of therapy (HR, 1.16; 95% CI, 1.06–1.26; P = .001). Among patients receiving CAPOX, 3 months of therapy was like 6 months of therapy (HR for DFS, 0.95; 95% CI, 0.85–1.06) and met the pre-specified margin for non-inferiority. Among patients with N1 tumors (<4 positive nodes), the HR was 1.07 (0.97–1.17), and among those patients with N2 tumors (≥4 positive nodes), the HR was 1.07 (0.96–1.19). Among patients with T4 tumors, a therapy duration of 3 months was inferior to a duration of 6 months (HR, 1.16; 95% CI, 1.03–1.31). Among patients with low-risk tumors (T1–3, N1), 3 months of therapy was non-inferior to 6 months of therapy (HR, 1.01; 95% CI, 0.90–1.12) with a 3-year DFS rate of 83.1% for patients who received 3 months of therapy and 83.3% for patients who received 6 months of therapy. Among patients with high-risk tumors (T4 or N2), 6 months of therapy was superior to 3 months of therapy (HR, 1.12; 95% CI, 1.03–1.23; P = .01). The IDEA study has generated much debate regarding the optimal length of therapy. It is

recommended that patients and doctors weigh the pros and cons of potential diminished efficacy of 3 months of therapy versus the definite increased risk of toxicity, particularly neuropathy. CAPOX appears to be slightly more active than FOLFOX in the adjuvant setting. The benefit of the doublet schedule with oxaliplatin and 5FU/LV has been demonstrated in two recent trials. In the MOSAIC study, the addition of oxaliplatin to 5-FU/LV (FOLFOX schema), showed a significantly increased disease-free survival (DFS) at 3 years, with a reduction in the risk of recurrence of 23% compared with the control arm (LV5FU2). The update at 6-year follow-up confirmed the benefit in DFS of adjuvant treatment with FOLFOX4 and an advantage was also observed in OS (absolute gain of 4.2%), but for stage III patients only. The NSABP C-07 trial compared the efficacy of bolus FU/LV + oxaliplatin (FLOX) versus FU/LV alone (Roswell Park schedule); 3-year DFS was 76.5% versus 71.6% for FLOX and FULV, respectively, and the magnitude of reduction in the risk of recurrence was similar to that of the MOSAIC trial. Spectrum of toxicity between MOSAIC and NSABP-C07 was different: grade 3–4 diarrhoea resulted higher with FLOX, while grade 3 sensory neuropathy was observed in 12% with FOLFOX and 8% with FLOX. As a result of these studies FOLFOX for 6 months has been adopted worldwide as the standard of care in stage III colon cancer patients.

c) **Capecitabine and Oxaliplatin (CAPOX)** CAPOX was evaluated in the adjuvant setting for patients with resected stage III colon cancer (capecitabine 1,000 mg/m2 bid on days 1 to 14 every 21 days and oxaliplatin 130 mg/m2 every 21 days for a total of 8 cycles). A randomized phase III trial (NO16968 [NCT00069121]), randomly assigned 1,886 patients with stage III colon cancer to receive CAPOX or bolus 5FU-LV (Roswell Park or Mayo Clinic schedule). The 7-year DFS rates were 63% for patients who received CAPOX and 56% for patients who received bolus 5-FU/LV (HR, 0.8; 95% CI, 0.69–0.93; P = .004). The 7-year OS rates were 73% for patients who received CAPOX and 67% for patients who received a bolus 5-FU/LV (HR, 0.83; 95% CI, 0.70–0.99; P = .04). On the basis of this trial, CAPOX has become an acceptable standard regimen for patients with stage III colon cancer. The X-ACT trial showed that in stage III capecitabine in monotherapy is an active agent with a favourable toxicity profile and may reduce overall costs compared with i.v. treatments [I]. After 4.3 years of follow-up the data still confirm the equivalence in terms of DFS between capecitabine and 5FU/LV. Capecitabine and oxaliplatin in combination have been evaluated in a range of different schedules and doses. The XELOXA international phase III study assessed the safety and efficacy of adjuvant capecitabine plus oxaliplatin (XELOX) versus bolus FU/LV (Mayo Clinic or Roswell Park regimen). The toxicity profile was different: patients receiving XELOX experienced less all-grade diarrhoea, alopecia, and more neurosensitive toxicity, vomiting and hand–foot syndrome. Preliminary data of efficacy, presented at the moment only as an abstract, indicated a benefit in DFS for XELOX. Also, the NSABP C-06 trial demonstrated the equivalence of oral treatment (UFT/LV) to 5FU/LV in stage II/III colon cancer patients but the drug is not approved in the adjuvant setting and therefore these data have no practical implication.

d) **Irinotecan** Negative trials are related to irinotecan in combination with 5FU (bolus or infusional). The CALGB-89803 trial compared 5-FU/LV + irinotecan (IFL) with

the Roswell Park scheme in >1200 patients. The trial was prematurely closed due to an elevated rate of mortality for IFL as compared with the FL regimen (2.2% versus 0.8%). Preliminary results indicated no improvement in terms of either OS or event-free survival for IFL, as compared with FL. The PETACC-3 trial compared the LV5FU2 or AIO regimen plus irinotecan with the LV5FU2 or AIO regimen alone. Results did not show an advantage for the regimen with irinotecan in terms of DFS. Also, the ACCORD trial, performed in high-risk stage III patients, did not report any significant benefit with irinotecan-based chemotherapy.

Unfortunately, in the adjuvant setting many questions are still unanswered. First of all, the role of targeted agents associated with chemotherapy. At the present time the AVANT study (bevacizumab + FOLFOX or XELOX versus FOLFOX) and the PETACC-8 trial in k-ras wild-type patients (cetuximab + FOLFOX versus FOLFOX) are exploring this question. At ASCO Meeting 2009 disappointing results were presented for the NSABP C-08 trial evaluating the addition of bevacizumab to FOLFOX: 3-year DFS was not improved by the biologic drug. Other ongoing trials in this field are the QUASAR 2 and E5202 (with bevacizumab) and the INT NO 147 (with cetuximab) trials. Second, the 'optimal duration' of adjuvant treatment: 3 or 6 months? In Italy, the TOSCA trial is investigating whether 3 months of FOLFOX4 treatment are not inferior to 6 months with the same schedule in terms of RFS in stage II and III colon cancer patients. Together with other studies, this trial is the backbone of a large international collaboration ('IDEA') which will give a definitive answer regarding the duration of adjuvant therapy in stage III patients. And last, the validation of prognostic/predictive factors: data are expected from large subset analysis in the context of international trials, such as PETACC-3, AVANT and PETACC-8 (98).

1.6.5 Stage IV CRC The optimal treatment strategy for patients with metastatic CRC (mCRC) should be discussed in a multidisciplinary expert team. In order to identify the optimal treatment strategy for patients with mCRC, the staging should include at least clinical examination, blood counts, liver and renal function tests, CEA and CT scan of the abdomen and chest (or alternatively MRI). The evaluation of the general condition, organ function and concomitant non-malignant diseases determines the therapeutic strategy for patients with mCRC. The general condition and performance status of the patient are strong prognostic and predictive factors. Known laboratory prognostic factors are white blood cell count, alkaline phosphatase level, lactate dehydrogenase, serum bilirubin and albumin. Additional examinations, as clinically needed, are recommended before major abdominal or thoracic surgery with potentially curative intent. An FDG-PET scan can give additional information on equivocal lesions before resection of metastatic disease, or can identify new lesions in the case of planned resection of metastatic disease. The majority of patients have metastatic disease that initially is not suitable for potentially curative resection. It is, however, important to select patients in whom the metastases are suitable for resection and those with initially unresectable disease in whom the metastases can become suitable for resection after a major response has been achieved with combination chemotherapy. The aim of the treatment in the last group of patients may therefore be to convert initially unresectable mCRC to resectable disease. The backbone of first-line palliative chemotherapy alone, as well in combination with targeted agents, consists of a fluoropyrimidine (FP) [intravenous (i.v.) 5-fluorouracil (5-FU) or the oral FP capecitabine] in various combinations and schedules]. Infused

regimens of 5-FU/leucovorin (LV) are less toxic than bolus regimens and should preferably be used. The oral FP capecitabine is an alternative to i.v. 5-FU/LV]. Combination chemotherapy with 5-FU/LV/oxaliplatin (FOLFOX) or 5-FU/LV/irinotecan (FOLFIRI) provides higher response rates (RRs), longer progression-free survival (PFS) and better survival than 5-FU/LV alone. FOLFOX and FOLFIRI as chemotherapy alone have similar activity and are both partners for biologicals, but have a different toxicity profile: more alopecia and, in most trials, more severe diarrhoea for irinotecan and more polyneuropathy for oxaliplatin. They also have potentially different interactions with biologicals. Both regimens consist of a 46- to 48-h administration every 2 weeks (q 2 weeks) with a bolus of 5-FU administration (LV5FU2) regimens. The dose of oxaliplatin in combination regimens with 5-FU/LV is between 85 and 130 mg/m² q 2 weeks; there is, however, no evidence that the dose at the higher range is more active. Therefore, a dose of 85 mg/m² is usually proposed. Four randomised studies have shown that combination chemotherapy was not superior to sequential treatment in terms of overall survival (OS), and therefore sequential therapy starting with FP alone remains a valid option in selected and frail patients for treatment with chemotherapy alone. Nevertheless, combination chemotherapy remains the preferred option as it allows better tumor growth control plus the option of de-escalation to FP alone. There are, however, no perfect selection criteria for determining which patients are still candidates for upfront FP therapy. It is estimated that today ~15% of patients are treated initially with an FP alone. The exposure to all three cytotoxics (FP, oxaliplatin and irinotecan) in various sequences may result in the longest survival, as a retrospective analysis indicates.The combination of capecitabine plus oxaliplatin (CAPOX; capecitabine 2000 mg/m²/day; day 1–14 q 3 weeks and oxaliplatin 130 mg/m² day 1 q 3 weeks) is an alternative to the combination of infused 5-FU/LV and oxaliplatin based on similar activity and safety profiles. The original 3-weekly regimen of capecitabine/irinotecan seems to be more toxic than 5-FU/LV/irinotecan. This regimen is therefore less well established and less frequently used. A dose-reduced regimen seems be less toxic, while maintaining the activity (capecitabine 1600 mg/m²/day for 2 weeks and irinotecan 200 mg/m² day 1 q 3 weeks). The data on triplet combination cytotoxic treatment with 5-FU, oxaliplatin and irinotecan are interesting, but remain controversial: an Italian randomised phase III study showed a better outcome for patients treated with FOLFOXIRI compared with FOLFIRI, while a Greek study did not show any difference. Second-line chemotherapy should be offered to patients with good performance status and adequate organ function. In patients refractory to an irinotecan-based regimen, second-line treatment must consist of an oxaliplatin-containing combination (FOLFOX and CAPOX). In patients refractory to FOLFOX or CAPOX, an irinotecan-based regimen is proposed as second-line treatment: irinotecan monotherapy (350 mg/m² q 3 weeks) and FOLFIRI are options. There is evidence that FOLFIRI has a better therapeutic index in second-line compared with irinotecan monotherapy, also because there are clear safety advantages of FOLFIRI compared with irinotecan q 3 weekly (89).

Targeted therapy in Stage IV disease Monoclonal antibodies (bevacizumab) or proteins (aflibercept) against vascular endothelial growth factor (VEGF) and against the epidermal growth factor receptor (EGFR) in combination with chemotherapy should be considered in patients with mCRC, since they improve the outcome of

mCRC. Only trials with a combination of cytotoxics and a biological targeted treatment consistently reported a median survival exceeding 24 months.

a) **anti-VEGF strategies**

Bevacizumab, an antibody that binds circulating VEGF-A, increases the activity of any active cytotoxic regimen. Bevacizumab has been shown to increase the survival, PFS and RR in first-line treatment in combination with 5-FU/LV/irinotecan and in combination with 5-FU/LV or capecitabine alone. Bevacizumab has also been shown to improve the PFS in combination with an FP plus oxaliplatin in the first-line treatment of mCRC. The combination of FOLFOXIRI plus bevacizumab has shown better PFS and RR than FOLFIRI plus bevacizumab in a trial with also one of the longest survivals reported to date. Bevacizumab is usually continued in combination with a cytotoxic agent/combination until progression or toxicity. Bevacizumab also improves the survival and PFS in combination with FOLFOX in second-line treatment. It has also been shown that continuing bevacizumab while changing the cytotoxic backbone, in second line after progression in first line, improves the outcome (survival and PFS). Bevacizumab has specific class-related side-effects: hypertension, proteinuria, arterial thrombosis, mucosal bleeding, gastrointestinal perforation and wound healing problems, but does not increase the chemotherapy-related side-effects. There are no validated predictive molecular markers available for bevacizumab.

Aflibercept, a recombinant fusion protein, that blocks the activity of VEGF-A, VEGF-B and placenta growth factor, improves survival, PFS and RR when combined in second line with FOLFIRI in oxaliplatin pre-treated patients, whether or not the patients were pre-treated with bevacizumab in first line. Aflibercept has a similar VEGF-related toxicity pattern compared with bevacizumab, but it increases the chemotherapy-related adverse events: diarrhoea, neutropenia, asthenia and stomatitis. Regorafenib is an oral multitarget tyrosine kinase inhibitor that has shown significant improvement of survival and PFS in patients refractory to all available cytotoxics and to bevacizumab and to the anti-EGFR antibodies; it can be proposed as a standard treatment in last line in fit and motivated patients with mCRC.

b) **anti-EGFR strategies**

In 1995, the first monoclonal antibody targeted to EGFR with convincing preclinical data was announced. The anti-EGFR antibodies cetuximab and panitumumab are active in different lines of treatment and in various combinations. It has been demonstrated that the (potential) benefit of anti-EGFR antibodies in all treatment lines and either as a single agent or in combination with any chemotherapy regimen is limited to patients in whom a RAS mutation is excluded. It was shown that the ‘expanded RAS’ analysis (also including the detection of mutations in exons 3 and 4 of the KRAS gene as well as mutations in the NRAS [exons 2–4] gene) is superior to the KRAS (exon 2) analysis in predicting both more efficacy in the expanded RAS

wild-type (WT) patients and a potential detrimental effect in patients harbouring any RAS mutation in their tumour genome. Therefore, the availability of an expanded RAS status is a prerequisite for any use of an anti-EGFR antibody. According to the European Medicines Agency (EMA), anti-EGFR antibodies must not be used otherwise. Named cetuximab, it is a chimeric immunoglobulin G (IgG) antibody that induces EGFR internalization and degradation once bound to the external domain of EGFR.77 Cetuximab showed great potential in progression-free survival (PFS) improvement in patients with low response to single-agent IRI therapy, according to the BOND trial, which contributed to the FDA approval of cetuximab for metastatic CRC in 2004. Moreover, a subsequent study also confirmed that cetuximab treatment prolonged OS and PFS in patients with CRCs when previous treatment with fluoropyrimidine, IRI and OX failed or was contraindicated.79 Combinations of cetuximab with other existing chemotherapies also displayed promising results. The phase III CRYSTAL trial found that cetuximab plus the FOLFIRI regimen had better progression control (8.9 vs. 8 months, hazard ratio (HR) 0.85; $p = 0.048$) than FOLFIRI alone, although the OS was not significantly different (HR, 0.93; $p = 0.31$).80 Interestingly, in different studies investigating cetuximab combined with FOLFOX in metastatic patients with CRC, no significant PFS or OS improvement was identified given that the doses in FOLFOX might have differed between studies because of the impact of the crossover design, but this lack of improvements in PFS and OS has also now been ascribed to CRC molecular heterogeneity. Maintaining cetuximab alone after a FOLFOX plus cetuximab regimen was not inferior to maintaining combination therapy in terms of PFS, with fewer adverse reactions noted. Escalating to the maximal dose of cetuximab based on the intensity of skin rash in the EVEREST trial suggested that an overall response might be achieved but without OS improvement. Murine-human chimeric antibodies might cause immunogenic reactions; therefore, the fully humanized antibody panitumumab has been developed, which does not trigger antibody-dependent cell-mediated cytotoxicity like cetuximab does87 and showed a lower risk of hypersensitivity reactions (0.6–3.0% for panitumumab and 3.5–7.5% for cetuximab). The efficacy of panitumumab against CRC was evaluated in the PRIME trial when FOLFOX plus panitumumab was compared with FOLFOX alone, and the combination regimen achieved a better PFS (10 vs. 8.6 months, HR 0.80, $p = 0.01$) and OS than FOLFOX alone (23.9 vs. 19.7 months, $HR = 0.88$, $p = 0.17$), with further demonstrated significance in the updated survival analysis ($HR = 0.83$, $p = 0.003$) in patients with metastatic CRC. Maintenance with panitumumab and 5-FU/LV after panitumumab plus FOLFOX showed numerical improvement in PFS and OS compared with single-agent panitumumab in the retrospective analysis of the PRIME and PEAK trials. The toxicity of this combination did not increase, which was confirmed in the VALENTINO trial, in which maintaining single-agent panitumumab appeared to have shorter PFS ($HR = 1.55$, $p = 0.011$) than treatment with panitumumab combined with 5-FU/LV. Cetuximab and panitumumab are both FDA-approved agents for the first-line treatment of CRC. No inferiority or superiority was identified in the phase III ASPECCT study between these two drugs. Cetuximab resulted in an OS of 10.0 months, and the OS was 10.4 months for panitumumab (HR 0.97, $p < 0.0007$ for noninferiority), in which no obvious adverse events were noted other than the incidence of grade 3 or 4 hypomagnesemia (3% for cetuximab and 7% for panitumumab). This also indicated that antibody-dependent cell-mediated cytotoxicity was not a major mechanism for these agents. However, in terms of quality-adjusted

life-years, panitumumab seemed to be more economically efficient than cetuximab. For second-line treatment of CRC or beyond, anti-EGFR agents might be low priority because in several studies cetuximab and panitumumab have been demonstrated to fail to reach statistically better PFS or OS for patients with CRC. In fact, only one study reported that panitumumab significantly prolonged PFS (8 vs. 7.3 weeks, $HR = 0.54$, $p < 0.001$) compared with best supportive care in patients with chemorefractory CRC with an acceptable rate of adverse events. In general, anti-EGFR agents are among the least attractive choices in second-line treatment, especially compared with anti-vascular endothelial growth factor (VEGF) agents, which will be discussed in a subsequent section. Notably, subgroup analysis has indicated that both of these anti-EGFR agents are robustly beneficial to those patients with RAS-wild-type tumors in the CRYSTAL, PRIME, and TAILOR trials, even though negative outcomes were experienced in patients with RAS mutations (KRAS and NRAS exon 2, 3, and 4 mutations). Interestingly, left-sided CRC tends to be more enriched for EGFR expression than right-sided CRC, in which MSI or BRAF mutations are predominantly activated. This sidedness leads to different clinical outcomes, such that worse OS and PFS have been observed in right-sided CRC than in left-sided CRC regardless of the choice of chemotherapy regimen or targeted agent. This biological factor has also been validated in anti-EGFR agent trials: in terms of RR, PFS, and OS within RAS-wild-type patients, those with left-sided tumors were expected to have better clinical outcomes than those with right-sided cancers. As demonstrated above, BRAF mutations are independent from RAS mutations and are closely related to a low anti-EGFR response, and both the NCCN and ESMO guidelines recommend using cetuximab and panitumumab in confirmed BRAF-wild-type and RAS-wild-type patients (89,99).

c) **anti-EGFR vs anti-VEGF strategy in RAS WT Stage IV disease**

To date, data from three head-to-head phase III studies are available: the AIO/FIRE-3 trial comparing FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab did not reveal a difference in RR (the primary end point) or in PFS in both the initially analyzed KRAS WT cohort and the RAS WT cohort, analyzed later. There was, however, a survival benefit (secondary end point) for patients treated with cetuximab compared with those treated with bevacizumab in the KRAS WT population, which was even more striking in the RAS WT population (HR 0.70) and a difference in RR according to an independent review of responses. However, the lack of a difference in ORR and PFS, and the limitation to the OS benefit as secondary end point, is not entirely understood. The same pattern [OS benefit in the RAS cohort (retrospective analysis, secondary endpoint) without difference in OS and PFS (primary endpoint)] was observed in a smaller phase II study comparing FOLFOX with panitumumab or bevacizumab. This phase III trial also had no formal hypothesis for comparison. Recently, results from the large (N = 1140) US Intergroup CALGB/SWOG 80405 study were reported, indicating no significant difference in OS (as the primary end point) if any chemotherapy (FOLFOX [in 73%] or FOLFIRI, according to investigator's decision) was combined with either bevacizumab or cetuximab (HR 0.925). There was also no interaction with the non-different OS with any type of chemotherapy. However, the currently available analysis of this trial is limited to patients with KRAS WT tumour; the analysis of RAS WT patients is expected in late 2014 and will be important in order to draw definitive conclusions on the best treatment option. Until then, all chemotherapy (FOLFOX/FOLFIRI)-antibody

combinations should be regarded as appropriate, and the decision-making will be a complex surrogate, taking into account many clinical factors, as well as patient preferences. Anti-EGFR antibodies should not be combined with bevacizumab (99).

Resection of metastatic liver disease Surgical resection of R0-resectable colorectal liver metastases is a potentially curative treatment, with reported 5-year survival rates of 20%–45%. The criteria for R0-resectability of liver metastases are not standardised and vary, depending on the experience of the multidisciplinary expert team. Resectability is not limited by number, size or bilobar involvement. The decision about technical resectability is based on several factors, including the rather 'technical' aspects like the possibility of performing R0 resection with sufficient remnant liver (>30%), including various surgical maneuvers, and the presence of resectable extrahepatic disease and co-morbidity of the patient. Moreover, the 'oncological' resectability should be added to the decision tree: criteria of the biology of the disease (e.g. synchronous versus metachronous, aggressiveness of the tumour and progression time) are important, but not easy to assess. Up to 75% of these patients will suffer a relapse following resection of their hepatic metastases, with the majority occurring in the liver. There is no role for partial palliative resection of metastases. Other ablative techniques, such as radiofrequency ablation or SBRT, may be added to surgery to obtain R0 resection or may be an alternative for resection in the case of poor anatomical localisation for resection, in order to keep enough remnant liver. Resection of resectable lung metastases also offers 25%–35% 5-year survival rates in carefully selected patients. Although resection of lung metastases is less well studied, R0 resection of lung metastases can also be recommended in analogy with resection of liver metastases. There are two potential strategies for (neo-)adjuvant therapy in patients with resectable liver metastases: postoperative adjuvant chemotherapy with FOLFOX for 6 months or perioperative chemotherapy (3 months before and 3 months after resection of the metastases). In patients with resectable liver metastases, perioperative combination chemotherapy with the FOLFOX regimen improves the PFS by 7%–8% at 3 years, although the survival is not significantly longer. The trials of modern postoperative adjuvant chemotherapy have many shortcomings, but it is suggested that an oxaliplatin-based chemotherapy for 6 months after resection of metastases improves the outcome, unless patients were failing an adjuvant treatment (oxaliplatin-based) for stage II or III diseases within 12 months. However, there are no data from randomised trials available to support this approach. The selection of perioperative chemotherapy or postoperative adjuvant chemotherapy may be influenced by the biology of the disease, the timing of metastases (synchronous versus metachronous) or the number and size of metastases. There is no evidence that adding a biological to a cytotoxic doublet improves the outcome in resectable metastases compared with a cytotoxic doublet alone in combination with resection of the metastases. Recent data even suggest that the addition of cetuximab to FOLFOX may be harmful to patients with resectable metastases. Initially unresectable liver metastases (group 1) can become resectable after downsizing with chemotherapy (conversion to resectable disease) and, if so, resection (±ablative techniques) should be considered after multidisciplinary discussions in an expert team. Pathological response seems to be a surrogate for predicting the outcome. Therefore, in patients with potentially resectable metastases, the goal has often been to achieve a high RECIST RR in order to convert unresectable metastases to technically resectable metastases. In patients in whom the metastases have disappeared on standard imaging,

microscopic disease is often still present and a multidisciplinary discussion of the optimal strategy has to take place. Surgery can be carried out safely after 3–4 weeks from the last cycle of chemotherapy ± cetuximab, or 6 weeks following chemotherapy plus bevacizumab. Resection of the metastases should be carried out as soon as the metastases are technically resectable, since unnecessary prolonged administration of chemotherapy may lead to increased liver toxicity and higher postoperative morbidity. The postoperative morbidity is more related to the duration of the chemotherapy than to the type of chemotherapy that is administered, although oxaliplatin and irinotecan may cause different histological changes in liver parenchyma: oxaliplatin is related to sinusoidal liver lesions and irinotecan to steatohepatitis.

Overall management The definition of a (potential) treatment aim is important for both the integration of a multimodal approach and for the choice of a first-line systemic treatment. Relevant factors are tumor- and disease-related characteristics, such as clinical presentation and patterns of tumor biology (e.g. metastases limited to liver and/or lung, dynamics of progression, symptoms and prognostic molecular or biochemical markers), as well as patient-related factors (co-morbidity and expectations of the patient). An established practical approach is to subdivide patients into four clinically defined groups:

a) Group 0: Primarily technically R0-resectable liver or lung metastases and no 'biological' relative contraindications (e.g. relapse during adjuvant treatment, etc.). Upfront resection is an option, specifically when metastases are limited in number and size. However, the only phase III trial in this situation has shown a benefit in disease-free survival and non-significant improvement of OS (51% at 5 years) if perioperative treatment with FOLFOX is administered.
b) Group 1: Potentially resectable metastatic disease with curative intention. The goal of a disease-free status after downsizing by chemotherapy, enabling secondary surgery, may give the potential of long-term survival or cure. Therefore, the most active 'induction' chemotherapy should be selected upfront in this group. Data from randomized trials suggest that the addition of a targeted agent to a cytotoxic doublet, or even to a triplet, may be the most effective combination, but FOLFOXIRI with and without bevacizumab also resulted in high RRs. To date, there are neither large randomized studies nor datasets of the head-to-head comparisons (FIRE-3, CALGB/SWOG 80405) in this specific cohort that would allow a definitive conclusion about which regimen should preferably be used. According to cross-trial comparisons in (K)RAS WT tumors with FOLFIRI/FOLFOX and to a prospectively planned assessment in the AIO/FIRE-3 trial , anti-EGFR antibodies appear to be more effective in terms of tumour shrinkage (and therefore, theoretically secondary resectability) than bevacizumab-based combinations . FOLFOXIRI ± bevacizumab can be an alternative option.
c) Group 2: Disseminated disease, technically 'never'/unlikely resectable intermediate intensive treatment. The treatment intention is rather palliative. In patients with symptoms, more aggressive biology or extensive disease, very active first-line treatment with a high likelihood to induce metastases regression in short time, seems to be the best option. In this group of patients, a cytotoxic doublet in combination with a targeted agent is generally proposed and should be regarded as the preferred option. The most often recommended targeted agent here is bevacizumab, in view of the continuum of care

approach, taking into account the fact that bevacizumab has only been examined in early lines (first and second line), the better subjective profile in terms of symptomatic toxicity, and that the activity of the anti-EGFR antibodies is at least as relevant in later lines compared with early lines. As an alternative option, in patients with RAS WT tumor first line therapy with FOLFOX or FOLFIRI plus an anti-EGFR antibody could also be considered, particularly in patients with relevant tumor-related symptoms, due to the earlier onset of response. The emerging data of head-to-head comparisons may refine the treatment schedule in this group of patients. In patients responding to the initially selected treatment, re-consideration of the treatment options should be done in a multidisciplinary team. In oligometastatic patients, ablative methods may be additionally considered, as they may allow a progression-free interval even without systemic treatment. In patients without the option for additional ablative treatment, a de-escalation of the initially selected combination may be considered. It is known that oxaliplatin combinations can be de-escalated to 5-FU/LV as maintenance treatment after a few months. For 5-FU/LV/oxaliplatin combinations with bevacizumab, two phase III trials have recently demonstrated that an active maintenance therapy (with fluoropyrimidines and bevacizumab) prolong PFS, without significantly improving OS compared with complete treatment discontinuation after 4.5–6 months. Therefore, active maintenance should be regarded as standard, although a complete discontinuation can be considered in suitable patients (e.g. with low tumor burden).

d) Group 3: Never-resectable metastatic disease—non-intensive/sequential treatment. For these patients, maximal shrinkage of metastases is not the primary treatment aim. Without present or imminent symptoms and limited risk for rapid deterioration, the aim is rather prevention of tumor progression and prolongation of life with minimal treatment burden. An intensive discussion with the patients on the benefit/risk ratio is important. Patients may be offered a combination cytotoxic ± a biological targeted agent, or an escalation strategy may start with an FP in combination with bevacizumab. On progression, appropriate strategies are to consider an oxaliplatin- or irinotecan-based combination (sequential approach) with a biological targeted agent. There are no randomized trials comparing the biological targeted agents in second line. In patients who started with bevacizumab (in combination with a cytotoxic doublet) in first line, the options are bevacizumab, aflibercept and, in RAS WT patients, the anti-EGFR antibodies such as cetuximab or panitumumab. Considerations for the choice include the choice of treatment in first line, the biology of the disease, the molecular characterization of the tumour, the time on first-line treatment (very short treatment on bevacizumab does not favor the continuation of bevacizumab), the toxicity of the agents, the knowledge of the activity of the anti-EGFR antibodies in later lines and the availability of the agents.

1.6.6 Resectable Rectal Cancer (RC)

For resectable rectal carcinoma the surgical principles of radical transabdominal resection for rectal cancer include: i) central ligation and division of the inferior

mesenteric artery (IMA). Although it has been claimed that there is not any significant difference in short-term outcomes and oncological results between a high tie of the IMA and a low tie after the origin of the left colic artery (100), current evidence shows that high IMA ligation and apical lymph node status assessment are of critical prognostic significance; ii) ligation and division of the inferior mesenteric vein just below the pancreas; iii) mobilization of the splenic flexure if necessary; and iv) Total mesorectal excision (TME) which involves en-bloc removal of the package of the rectum and mesorectum covered by their intact embryologic envelop, that is the posterior mesorectal fascia and the Denonvillier's fascia. This is achieved by sharp dissection in the well-confined embryological planes and by preserving the autonomic pelvic nerve plexuses (101–103). The macroscopic assessment of the quality of the resected specimen according to specific definitions is mandatory (104). A complete TME specimen with intact fascia and no coning towards the bowel wall (intramesorectal or muscularis dissection) is a strong positive prognostic factor of local recurrence prevention [79], as is the negative by 1-2 mm CRM (105). The distal to the tumor transection of the rectum is achieved either transabdominally or transanally, and the colo-anal anastomosis is fashioned with the use of a circular stapling device or by hand respectively. A temporary defunctioning stoma to protect the anastomosis is strongly recommended, particularly in case of a very low colo-anal anastomosis, an anastomosis in the obese male patient, and after neo-adjuvant treatment (106). The defunctioning stoma can be closed 3-6 months later, provided anastomosis is complete and leak is not identified by proctoscopy or double contrast imaging. For tumors located at the upper third of the rectum and the rectosigmoid junction a high anterior resection of is recommended. For tumors located in the middle rectum (6-10 cm from the anal verge), LARR with TME, and preservation of the pelvic nerve plexuses is indicated. A clear distal bowel margin of at least 1 cm is required. However, a distal margin <1 cm may be adequate, provided that pathology report confirms a negative margin and CRT has preceded surgery (107). For T1, N0 tumors or T2-3, N0 subjected and responding to CRT and in which a distal bowel clearance >1 cm does not involve a major part of the external anal sphincter, a LARR with TME and intersphincteric distal dissection with hand-sewn colo-anal anastomosis is recommended (108). Intersphincteric resection for low and ultra-low rectal cancer is associated with low morbidity, local recurrence rate of approximately 7%, disease-free survival of 78% and acceptable functional results (109).

Chemoradiotherapy for rectal cancer treatment (Preoperative treatment)

The aim of preoperative treatment is to reduce the risk of local relapse, to improve resectability and to enable R0 resection in CRM-positive disease. There are two approaches to preoperative therapy: short-course radiotherapy (RT) (25 Gy in 5 fractions) and long-course (50.4 Gy in 28 fractions) RT combined with chemotherapy (ChT). Most series suggest that there is improved outcome with increasing pathological response to CRT with patients achieving a pathologic complete response (pCR) having a local recurrence risk of 1.5% and overall survival over 90% (110). A series of retrospective studies from Brazil has highlighted the rationale of a 'wait-and-see' policy for patients who achieved a pCR (111), but long-term prospective observational studies with more uniform inclusion criteria are required to evaluate the risk versus benefit of this policy. It has been shown (CAO/ARO/AIO-94 trial) that

preoperative CRT followed by adjuvant ChT compared to postoperative adjuvant CRT significantly reduces local recurrence, has less acute and long-term toxicity and in addition enables a higher rate of sphincter saving surgery by downsizing and thus improves functional outcome in low rectal tumors (112,113). Therefore, preoperative CRT is the treatment of choice for all patients at higher risk for relapse (clinical stage II/III).

A) **Short-course RT** A growing body of evidence suggests that short-course RT versus surgery alone for resectable rectal cancer leads to a significant decrease in local recurrence (114). Two most recent large studies, the Dutch TME trial and the MRC CR07 trial (115,116), confirmed the significant benefit on local control with short-course preoperative RT even with TME surgery. Both trials showed no benefit in survival, however, in the MRC CR07 trial 3-year disease-free survival was improved in the preoperative irradiated group. Acute toxicity after short-course RT is usually mild when surgery is not delayed and, although early trials revealed a significant increase in late toxicity, this has not been reported in the more recent studies.

B) **Long-course Chemoradiotherapy (CRT)** Very limited tumor shrinkage and no tumor downstaging is expected after short-course RT and immediate surgery, while after the long-course CRT schedule the longer overall treatment time and interval to surgery results in downsizing of virtually all cancers and in pCR in approximately 15-20% of patients. Therefore, CRT is indicated in locally advanced T3c, d, T4, N+ disease and cases with a threatened or involved CRM. The same approach is recommended for upper rectal or rectosigmoid tumors that invade adjacent structures. MRI staging may allow a selection of patients with early stage III disease (stage T3a/b) in whom preoperative treatment may not be necessary (117). Standard long-course CRT regimens include 3D conformally planned RT for 5.5 weeks (50.4 Gy total dose) and either continuous infusion of 5-fluorouracil (5-FU) or capecitabine per os. Targeted chemotherapeutic agents are not recommended in this setting.

C) **Short- vs. long-course CRT** Three randomized trials of preoperative short- versus long-course CRT have been reported in patients with resectable T3/T4, any N rectal cancer (118,119). Both trials showed higher rates of early RT toxicity in the CRT groups but no significant differences in late toxicity. In the Polish study (118), the sphincter preservation rate, the local recurrence rate and overall survival did not differ significantly between the two groups. In the Australian trial (118), preliminary analysis also showed no significant differences in local control or survival. The Norwegian study (119) showed that long-course CRT is associated with better local control of the disease and higher survival rates compared to short-course RT. However, the question of which schedule is superior has not been resolved since the trials are small and underpowered to detect small differences, and longer follow up is required.

D) **Upfront ChT prior to surgery** Intensive combination ChT, with 5-FU and oxaliplatin (FOLFOX) combined with targeted agents has been proposed instead of neo-adjuvant CRT prior to surgery in T3 tumors that do not threaten the circumferential mesorectal margin, as it achieves a pCR in more than one

fourth of the cases. This approach is not recommended outside clinical trials, because of limited available data.

E) **Upfront ChT prior to preoperative CRT** In locally advanced rectal tumors, intensive and ChT with or without the addition of targeted agents prior to neo-adjuvant treatment and surgery has been associated with increased R0 rate and reduced rate of metastatic disease (120,121). However, data are limited and the approach should not be applied outside investigational clinical trials.

Non-operative approach after combined modality therapy (CMT) for Rectal Cancer Following neo-adjuvant CRT for locally advanced rectal cancer, pathology complete response (pCR) with no residual tumor at surgery is observed in 13-25% of the cases (122). Also, there is substantial evidence that patients with pCR after CMT, subjected to TME, show excellent oncological outcomes, with a local recurrence rate of only 0.7%, distant metastasis rate of 8.7%, overall survival of 90% and disease-free survival of 87% (109). Considering that definitive surgical treatment is associated with significant morbidity and that patients with pCR after CMT are of good prognosis, "expectant policy" and "deferring surgery" in case of recurrence could be a rational policy. If patients with pCR are approached non-operatively and observed closely, local and distant recurrence are seen in only 0-1.6% and 0-8.9% respectively (123). However, opposite results concerning recurrence after initial pCR show rates greater than 80% within the first year (124). Therefore, non-operative management should be reserved only for those with durable pCR. At present, non-operative treatment in patients with pCR after CRT should be applied in research protocols, and be reserved for patients unfit or unwilling to undergo surgery.

Interval from end of neo-adjuvant treatment and definitive surgery In case of neo-adjuvant treatment in the form of short-course RT, the time interval of surgery is 1-2 weeks (118,125). Following a long course of CRT, the exact time interval to surgery has not been defined, and varies from 6 to 12 weeks (126). This depends on the grade of tumor response to neo-adjuvant treatment. Even if a complete response (CR) is detected on pelvic MRI 6 weeks after the end of treatment, this should be followed by resection of the rectum. Deferral of surgery in case of CR is only allowed within the frame of a research protocol ("wait-and-watch" or "expectant" policy). In case of locally advanced (≥T3c/T4) rectal cancer, neo-adjuvant CRT aims to downsize and downstage the local disease (127). If response is favorable as assessed by pelvic imaging, curative resection (R0) can be achieved. There is good evidence that patients with pCR after CRT show significantly lower local recurrence and higher survival rates than those with partial response (128).

Very early rectal tumor

Although evidence is limited and well-designed clinical trials are required for cT1, sm1 tumors with low-risk features, standard transanal excision or excision by means of TEM, if technically feasible, is recommended as definitive treatment. For cT1, sm2 tumors with low-risk features definitive treatment (TME) is the treatment of choice. Local excision is not recommended outside clinical trials, unless the patient refuses definitive treatment or has co-morbidities (129). If histopathology shows deeper

invasion or additional high-risk features (poor differentiation, lympho-vascular, venous or neural invasion) CRT can be added with or without definitive surgery.

Early rectal tumor

For cT1, sm3 and T2 tumor neo-adjuvant treatment is not necessary and definitive transabdominal surgery with TME is recommended. cT3a, b, N0, CRM (-) or non-threatening tumors of the middle and upper rectum are treated either with upfront short-course RT followed by transabdominal TME or with transabdominal TME alone. According to the CR07 trial, there is a marginal but significant benefit, in terms of local recurrence for early rectal tumors even of the upper rectum after preoperative short-course RT, but long-term morbidity must be taken into account (115). If quality of surgery has been compromised or pathology shows positive CRM and neo-adjuvant treatment has not been given, postoperative CRT is recommended, with the expense of rather poor functional results.

More advanced rectal tumor

For cT3c/d, or cT tumors of the middle and upper rectum non-threatening or not involving the CRM, EMVI(-) preoperative treatment, either short-course RT or long-course CRT, followed by transabdominal TME surgery is recommended. In this group, cT3a/b tumors of the lower rectum, non-threatening or not involving the CRM, EMVI(-) are included. Also, preoperative treatment, either short-course RT or long-course CRT, followed by transabdominal TME surgery is recommended. There is not enough evidence in favor of either neo-adjuvant approach, short-course RT or long-course CRT. The former is less costly and is associated with acute toxicity; the latter can achieve significant down-staging and down-sizing.

Locally advanced rectal tumor

For cT3, CRM (threatening or +), or/and EMVI(+) and T4 tumors, neo-adjuvant long-course CRT is recommended. If response is favorable and R0 is possible, transabdominal TME -and beyond if necessary- surgery is recommended. Otherwise, palliative measures are recommended (see below). In this group, patients with involved lymph nodes of the lateral pelvic wall (obdurator, internal ileac nodes) are included, and neo-adjuvant CRT with extended lateral field is recommended. Surgery follows, if a R0 resection can be achieved by either TME or/and more extended pelvic surgery including removal of the lateral pelvic lymph nodes (130).

1.7 Biomarkers

The term “biomarker”, a portmanteau of “biological marker”, refers to a broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly. There are several more precise definitions of biomarkers in the literature, and they

fortunately overlap considerably. In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention."(131,132). A joint venture on chemical safety, the International Programme on Chemical Safety, led by the World Health Organization (WHO) and in coordination with the United Nations and the International Labor Organization, has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (133). An even broader definition takes into account not just incidence and outcome of disease, but also the effects of treatments, interventions, and even unintended environmental exposure, such as to chemicals or nutrients. In their report on the validity of biomarkers in environment risk assessment, the WHO has stated that a true definition of biomarkers includes "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction." (134). Therefore, biomarkers are by definition objective, quantifiable characteristics of biological processes. The use of biomarkers, and in particular laboratory-measured biomarkers, in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined. The key issue at hand is determining the relationship between any given measurable biomarker and relevant clinical endpoints. A molecular biomarker is defined as a biological molecule which can be termed as measurable indicator found in blood, and other body fluids, or tissues by which a particular pathological or physiological process, or of a condition or disease can be identified. In this regard molecular biomarkers can be utilized for diagnostic, prognostic and/or predictive purposes in the clinical or pre-clinical setting. A diagnostic biomarkers are used for risk stratification and early detection of colorectal polyps (135). A prognostic biomarker provides information about the patients overall cancer outcome (likelihood of a clinical event, disease recurrence or progression), regardless of therapy, whilst a predictive biomarker gives information about the likelihood that an individual with the biomarker experiences for a favorable or unfavorable effect of a therapeutic intervention than similar individuals without the biomarker (136,137). Examples of currently used prognostic and predictive biomarkers are described in Table 2.

Predictive		**Prognostic**	
KRAS , NRAS mutation	Negative response to the anti-EGFR (epidermal growth factor receptor) antibody-based therapies, cetuximab and panitumumab (138–140)	MSI	MSI-H is associated with better survival rates than both MSI-L and MSS (141,142)
BRAF V600E mutation	anti-EGFR inhibitor antibody resistance	BRAF V600E mutation	Poor prognosis in right-sided MSS CRC (143,144)
PIK3CA mutation	Resistance to cetuximab	Preoperative carcinoembryonic antigen (CEA) levels	Increased preoperative CEA (>5 ng/ml) correlates with poor prognosis (145)
PTEN (Phosphatase and tensin)	Shorter progression-free survival in patients with primary or metastatic CRC treated with	Cancer antigen 19-9 (CA 19-9) levels	Increased CA 19-9 present more frequently metastases thus making it a marker of poor prognosis

homolog protein deficiency	cetuximab-based therapy		(146)

Table 2. Examples of currently used prognostic and predictive biomarkers along with the clinical utility

1.7.1 Carcinoembryonic antigen (CEA) CEA are membrane-associated glycoproteins playing a number of roles in cell adhesion or signal transduction. Monoclonal CEA (mCEA) may be expressed in a wide variety of adenocarcinomas, including those arising from the colon. Although this lack of specificity limits its value as a diagnostic marker of CRC, it remains a useful component of a broad diagnostic panel. In a meta-analysis study by Tan et al based on 20 different studies, serum CEA has been demonstrated to comprise an exam of elevated specificity, although sensitivity was inadequate when tracing CRC recurrent conditions. The cut-off range varied among these studies from 3 to 15 ng/ml, suggesting that a measure of 2.2 ng/ml would be the optimal regarding sensitivity and specificity (147). Evidently, at this point circulating CEA may constitute a primary means of recording progress in patients' surgical follow-up, in accordance with the complementary tools including clinical picture, radiological response and histological results. CEA may be tested in the preoperative setting in patients with CRC in order to assist staging and surgical treatment planning. Previous findings showed that increased preoperative CEA (>5 ng/ml) correlates with poorer prognosis. In fact, a study with 2,230 patients proved that pre-operative CEA levels was an important independent prognostic factor when outcome prediction was encountered (148). Similarly, another study with 1,146 patients with CRC found that, following use of a multivariate analysis, preoperative CEA levels proved a highly significant prognostic factor even when stage and grade were introduced in the model (149). In addition, elevated preoperative CEA in stage III and IV CRC are considered to be a potent independent risk factor as far as local relapse, short disease-free survival and OS are concerned. After surgical removal of the tumor, CEA levels should be checked as it is shown that persistent by elevated CEA levels suggest further evaluation for metastatic disease. In addition, elevated CEA is very efficient for revealing recurrence in asymptomatic patients and is the most sensitive detector for liver metastases

1.7.2 KRAS and NRAS The family of RAS proteins (H-, K-, and N-RAS) is located in the intracellular side of the cell membrane involved in G-protein mediated signal transduction. Activation of the epidermal growth factor receptor (EGFR) from its ligand (e.g., EGF, TGF-α, amphiregulin) results in a change from GDP- to GTP-form of the KRAS, leading to increased concentrations of B-rapidly accelerated fibrosarcoma (proto-oncogene) (BRAF) to the plasma membrane. BRAF activates the mitogen-activated protein kinases (MAPK) signaling pathway that results in the expression of proteins involved in several pathways with a crucial role including cell proliferation, differentiation, survival, angiogenesis, and cell motility (Figure 4).

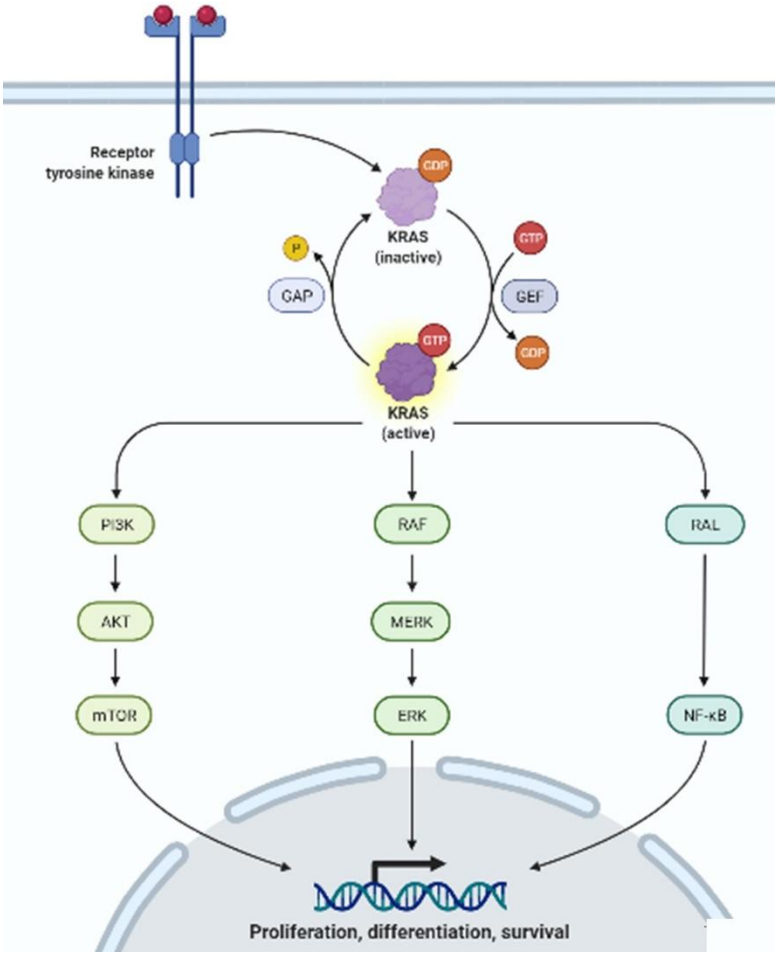

Figure 4. Graphical presentation of the KRAS-PI3K-AKT-mTOR, KRAS-BRAF-MERK (MAPK)- ERK (MAPKK), KRAS-RAL-Nf-κB pathways. Created with BioRender.com

Mutant KRAS proteins present as locked in the active form as a result of an impaired GTPase activity, leading to an increased proliferating rate that is unregulated and thus resulting in an overall malignant transformation of the cells. Therefore, it is logical to assume that mutation of these oncogenes poses a great threat for carcinogenesis). Indeed, it is well documented that alterations of KRAS gene can act as the first step towards carcinogenesis in approximately 50% of the CRC cases. However, it is not that clear whether it could serve as a prognostic marker in the clinical setting. Recently, a meta-analysis of seven studies failed to associate KRAS mutation status with prognosis (however, a common limitation of such studies is the small pool of patients) (150). On the other hand, two large multicenter studies, demonstrated that only one mutation of KRAS, of codon 12, could be linked with a more aggressive progression of cancer cells. However, according to their data, KRAS mutations failed to be associated with tumor location or stage and recurrence of disease. On the other hand, the possible prognostic value of NRAS mutations is less examined, even though they appear to act in a similar way with KRAS as for the degree of negative prognostic significance. Mutations of KRAS gene have proven their clinical use as a predictive biomarker in response to the EGFR inhibitors clinically used, as various mutations of KRAS present resistance to therapy with EGFR receptor monoclonal antibody blockers such as cetuximab. In detail, anti-EGFR therapy on cases of metastatic CRC who display KRAS mutations either of the codon 12 or 13, present no

benefit (151). However, a mutant KRAS does not always mean that it is a sign of bad response. For example, De Roock et al found that patients with metastatic CRC with P.G13D KRAS mutation treated with cetuximab exhibited better results than other KRAS mutations (152). Under the light of these findings, evaluation of an extended panel of RAS mutations including mutations in KRAS exon 2, 3 and 4 and NRAS exons 2, 3, and 4 can better discriminate which patients are not good candidates for treatment with anti-EGFR therapy

1.7.3 p53 p53 is a transcription factor that participates in a variety of cellular reactions to several stress situations such as mutagenic DNA damage, oncogene activation, hypoxia and telomere shortening. In contrast to other mutations, p53 mutation seems to occur late in the development of CRC since few or even no cases of mutations are described in precancerous lesions from sporadic adenomas and polyps. Interestingly, data from one study showed that mutations of p53 in CRC exhibits a dependence on the primary tumor site thus suggesting a prognostic value of this marker. In more detail, patients with a primary tumor site in the proximal colon (caecum, ascending colon) and mutant p53 gene exhibited better survival when treated with a combination of chemotherapy and surgical removal compared to those treated by surgery alone (153).

1.7.4 Phosphoinositide 3-kinase (PI3K) PI3K is a downstream mediator of the EGFR signaling cascade along with AKT and PTEN. An interesting finding regarding PI3K mutations lies on the use of cetuximab in patients with CRCs who display mutations of PI3K and especially at the PIK3CA exon 20 (which is the kinase domain) in contrast to patients with PIK3CA-wild-type CRCs. Patients with mutant PI3K exhibit much worse results. However, the mutation of a different exon, PIK3CA exon 9 (which is the helical domain), cannot serve as a predictive marker for anti-EGFR therapy, a fact that reflects the high complexity of the effects of specific mutations on different functions of the mutant kinases (154).

1.7.5 BRAF BRAF is serine-threonine protein kinase that is recognized downstream in the KRAS signaling cascade. BRAF mutations are linked with a poor outcome thus proving their clinical applicability as a prognostic marker in the adjuvant setting. Another indication of a poor outcome is found in stage II and III CRC cases where BRAF mutations were associated with worse overall survival. A rather interesting finding is that BRAF V600E mutation is able to predict a poor prognosis in right-sided MSS CRC. Further examination of the BRAF prognostic value was carried with a study investigating the correlation of BRAF mutation with MSI. The existence of BRAF wild-type and MSI-H exhibited favorable outcomes. Furthermore, BRAF-wild/MSS and BRAF-mutated/MSI-H exhibited intermediate outcomes. BRAF mutations and especially BRAF V600E is among the most common mutations found in CRC cases as it is present in approximately 8–10% of all cases. BRAF mutation is often used as a discrimination factor between familial and sporadic CRC as the existence of BRAF V600E mutation in MSI CRCs can virtually exclude Lynch syndrome (155). However, numerous studies have shown that tumors that exhibit BRAF mutation are resistant to anti-EGFR therapy.

1.7.6 Microsatelite instability (MSI) Microsatellites (MSs) are short tandem-repeated base pairs of 1–6 scattered all over the genome. For the normal human genome the number of MSs is approximately half a million. Genome studies revealed that MSs are prone to duplication errors. However these errors are usually corrected

by the MMR system. Consequently it is logical to assume that a defective MMR system would result in the accumulation of DNA mistakes and thus MSI. Indeed, MSI arises by the inhibition of MMR system either via defective methylation of MLH1 in CpG island or point mutation of any MMR genes (hMLH1, hMSH2, hMSH6, PMS1 and PMS2) especially hypermethylation of hMLH1 promoter. It is estimated that about 15–20% of CRC patients present MSI with a small fraction of which 2–4% are related to hereditary non-polyposis colorectal cancer (HNPCC). In order to estimate MS status, Bethesda panel was agreed in which five MS loci were included (BAT25, BAT26, D5S346, D2S123, and D17S250). However, some researchers suggested an expanded Bethesda panel include 10 loci. Based on this panel, MSI can be divided into three groups: MSI-high (MSI-H), defined as having ≥30% unstable loci using mononucleotide or dinucleotide markers; MSI-low (MSI-L), with 10–30% unstable loci; and microsatellite stable (MSS), with <10% unstable loci (156). MSI status varies according to a given CRC stage: Stage II CRC exhibits high prevalence of MSI (20%) while in stage IV CRC MSI is approximately 4%. Moreover, differences based on the MSI status are found when prognosis is examined. For example, cases with MSI-H CRC share a better prognosis than that with MSS CRC. MSI status is a well-studied diagnostic marker for CRC as mentioned above. However, its clinical relevance does not stop in the diagnostic setting but continues in the prognostic as well. MSI-H is associated with better survival rates than both MSI-L and MSS, not only in HNPCC, but also in sporadic cases. Recent studies have investigated the application of level of MSI status as a potential predictive marker of adjuvant therapy. While there is enough evidence supporting that MSI-H may predict response to 5-fluorouracil (5-FU)-based adjuvant therapy in stage III colon cancer, numerous recent studies demonstrated that there is no significant difference between patients with MSI-H and MSS tumors when 5-FU-based adjuvant therapy is used. This finding is very important for the group with stage II disease, in which adjuvant chemotherapy (5-FU alone) is reported to improve survival by approximately 3%, allowing some investigators to suggest that stage II colon tumors should be analyzed for MSI status as well in order to assist guide decisions on the use of adjuvant therapy.

1.7.7 Cancer antigen 19-9 (CA 19-9) CA 19-9, also termed sialyl Lewis a, is a documented marker with prognostic value for CRC. It is shown that cases with increased CA 19-9 present more frequently metastases thus making it a marker of poor prognosis (157). Similarly, a recent study with stage IV CRC proved that the preoperative serum CA 19-9 level can be a promising marker of tumor recurrence and prognosis in cases submitted to curative resection. In detail, high levels of CA 19-9 were connected with worse 3-year relapse-free survival and 3-year overall than that in the normal CA 19-9 group.

1.8 Micronuclei

1.8.1 Milestones in Micronuclei Formation Micronuclei (MN), also known as Howell-Jolly bodies, are tiny extra-nuclear bodies originating from acentric chromatid/chromosome fragments or whole chromatids/chromosomes that lag behind at the anaphase of dividing cells and are not included in the main nucleus during telophase. Instead, they are enwrapped by the nuclear membrane and resemble the

structure of the daughter nucleus, although being way smaller in size. Acentric chromatid/chromosome fragments usually originate after extensive DNA damage such as DSBs that if misrepaired result in asymmetrical chromosome rearrangements and exchanges. Whole chromatids or chromosomes in MN are formed due to deficiencies in chromosome segregation during anaphase usually caused by mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation, and defects in the cell cycle control system. To form an acentric fragment, DNA double-strand breaks (DSBs) should either occur in one sister chromatid or extend to the whole anaphase chromosome. This happens only if the level of DSBs exceeds the repair capacity of dividing cells, which is mainly due to either the misrepair of DSBs by the dysfunctional homologous recombination or defects in enzymes of the non-homologous end-joining (NHEJ) pathway. The formation of DNA DSBs and MN is often the result of simultaneous excision repair of damages and wrong base incorporation. A failure of the appropriate gap-filling event leads to DSB. Malsegregation of sister chromatids usually happens due to the absence or inappropriate attachment of spindle microtubules to chromosome kinetochores. Stable amphitelic microtubule attachments generate tension at kinetochores, locking the correct chromatid orientation in place. Unstable microtubule–kinetochore attachments such as syntelic (both sister chromatids are attached to the same spindle pole), monotelic (only one kinetochore is attached leaving the second sister chromatid unattached), or merotelic (one kinetochore is attached to both spindle poles) do not result in significant tension, thus making the bond sensitive to dissociation. If not corrected, such attachments lead to inappropriate segregation and chromosome loss, thus resulting in aneuploidy and micronucleus formation, respectively. Sometimes, chromatids/chromosomes are unable to segregate as the mitotic spindle cannot pull them apart due to tubulin depolymerization. The absence of kinetochore or centromeric defects also lags chromosomes behind at telophase. Nucleoplasmic bridges and nuclear buds (NBUDs) are similar to MN. NPBs originate from dicentric chromosomes – ones that have two centromeric regions. Dicentrics are products of either HR between complementary DNA sequences of different chromosomes or NHEJ between two chromosomes that suffer from DNA DSBs. Similarly to the latter, NHEJ can be recruited to improperly encapsulated telomeres during their shortening. If a 3′ single-stranded overhang at the telomere is not properly capped, it is recognized as a broken DNA molecule which should be fused by NHEJ mechanism. Having two centromeres, a dicentric chromosome may attach to two opposite spindle bodies which pull chromatids in the opposite directions. In the absence of breakage, the nuclear membrane surrounds both nuclei forming NPBs between them. Eventually, NPB is broken during cytokinesis, resulting in a micronucleus formation. Misrepair of two chromosomal breaks causes the formation of both dicentric and acentric fragments. The latter will form MN of its own. Telomere fusion results in NPB that after breakage will accompany one of the daughter nuclei in the form of MN. MN and NPBs formed after telomere fusion contain telomeric sequences which can be recognized by specific probes that hybridize to subtelomeric regions. In contrast, MN and NPBs originated from DSBs misrepair are telomere negative. Anaphase bridges are initial events in breakage-fusion-bridge (BFB) cycles which are the features of chromosomal instability. The uneven breakage of NPBs leads to the formation of two daughter nuclei, one of which gained extra genetic information, whereas the second one lost an equal amount of genetic information. Such broken chromosomes usually do not contain telomeric zones and, therefore, can fuse with

their replica during the next mitotic event, repeating the cycle for the next couple rounds. BFB cycles lead to the amplification of genes near the break point which are eventually looped out of the abnormal chromosome, thus forming the so-called double minutes (DMs). DM chromosomes are selectively located at the periphery of the nucleus and are eliminated from the nucleus by nuclear budding during S phase. A NBUD is virtually the same as a micronucleus, except for its closer location and connection to the nucleus through a narrow cytoplasmic passage. NBUD also contains interstitial or terminal fragments without centromeric or telomeric regions, whereas MN is formed from a lagging chromosome. While briefly summarizing the mechanisms of MN formation, it is important to emphasize that MN containing acentric chromatids or chromosomes are the result of unrepaired or misrepaired DNA breaks, whereas MN with whole chromatids/chromosomes are formed due to (a) hypomethylation of satellite centromeric/paracentromeric sequences, (b) kinetochore defects, (c) dysfunctional spindle, and (d) mutations in anaphase checkpoint genes. NPBs originate from misrepaired DNA breaks, telomere end fusion, or failure of sister chromatids to separate due to the lack of decatenation. Last but not least, nuclear budding occurs as a result of either elimination of amplified DNA resulted through BFB cycles or specific elimination of excess chromosomes in polyploidy cells (158). It is suggested that the possible future that a micronucleus may have is: a) eradication of the whole cell through cellular apoptosis, b) ejection from the cytoplasm in case that the DNA it contains is not functional, c) re-integration into the main nucleus and d) maintenance in the cytoplasm as a distinct structure in case that the micronucleus has the mechanisms to divide separately from the main nucleus.

1.8.2. Cytokinesis-Block Micronucleus Assay in Lymphocytes The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MN in cultured human and/or mammalian cells because scoring is specifically restricted to once-divided BN cells, which are the cells that can express MN. In the CBMN assay, once-divided cells are recognized by their BN appearance after blocking cytokinesis with cytochalasin-B (Cyt-B), an inhibitor of microfilament ring assembly required for the completion of cytokinesis (Figure 5).

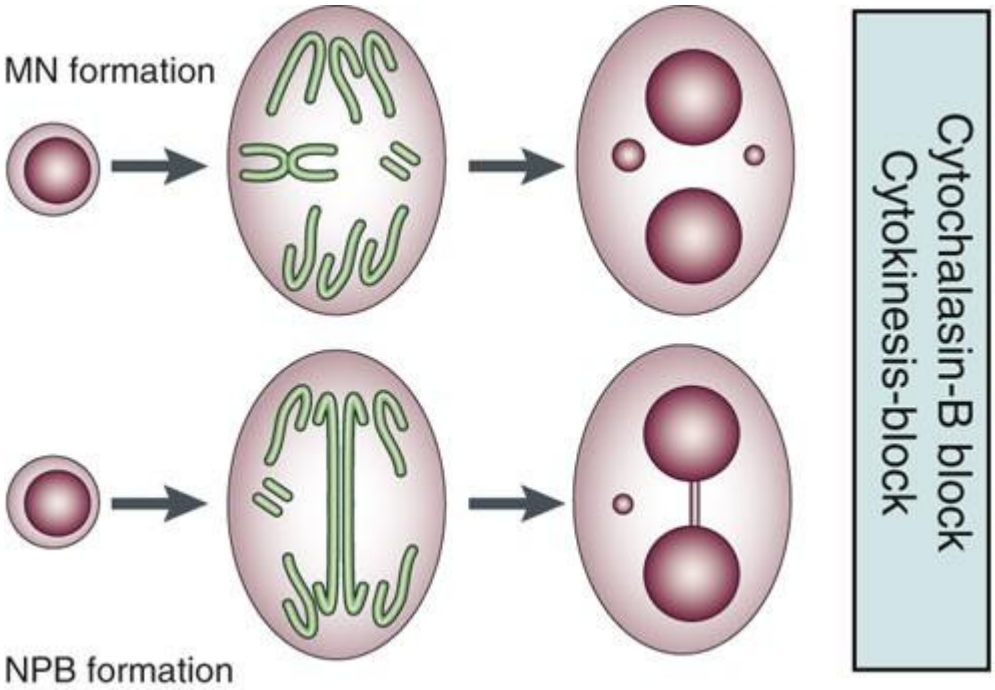

Figure 5 MN and NPB formation in cells undergoing nuclear division: MN originate from either lagging whole chromosomes or acentric chromosome fragments. NPBs originate from dicentric chromosomes that may be caused by misrepair of double strand DNA breaks or telomere end fusions. These events can only be observed in cells completing nuclear division, which are recognized by their BN appearance after cytokinesis blocking with Cyt-B. (Adopted by Fenech 2007)

Restricting scoring of MN in BN cells prevents confounding effects caused by suboptimal or altered cell division kinetics, which is a major variable in micronucleus (MN) assay protocols that do not distinguish between non-dividing cells that cannot express MN and dividing cells that can. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicology testing in human and mammalian cells. Table 3 presents the information that should be registered during CBMN evaluation.

Information that should be registered during CBMN evaluation
1. The code number of the specimen under evaluation
2. The number of binucleated cells counted
3. The number of binucleated cells with 0, 1, 2 or 3 micronuclei
4. The total number of micronuclei in binucleated cells
5. The total number of micronuclei per 1000 binucleated cells
6. The total of binucleated cells with micronuclei per 1000 binucleated cells
7. The total number of cells with 1, 2, 3 and 4 nuclei every 2000 cells
8. The cytokinesis block proliferation index (CBPI)

Table 3. Information needed for comprehensive CBMN evaluation

The CBMN assay is a comprehensive technique for measuring DNA damage, cytostasis, and cytotoxicity in different tissue types, including lymphocytes. DNA damage events are scored specifically in once-divided binucleated cells. These events include; (a) micro-nuclei (MNi), a biomarker of chromosome breakage and/or whole

chromosome loss; (b) nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions; and (c) nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects are measured via the proportion of mono-, bi-, and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios. The assay has been applied to the biomonitoring of in vivo exposure to genotoxins, in vitro genotoxicity testing and in diverse research fields, such as nutrigenomics and pharmacogenomics. It has also been shown to be important as a predictor of normal tissue and tumor radiation sensitivity and cancer risk. Figure 6 illustrates the various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents while Figure 7 illustrates photomicrographs of the cells scored in the CBMN assay.

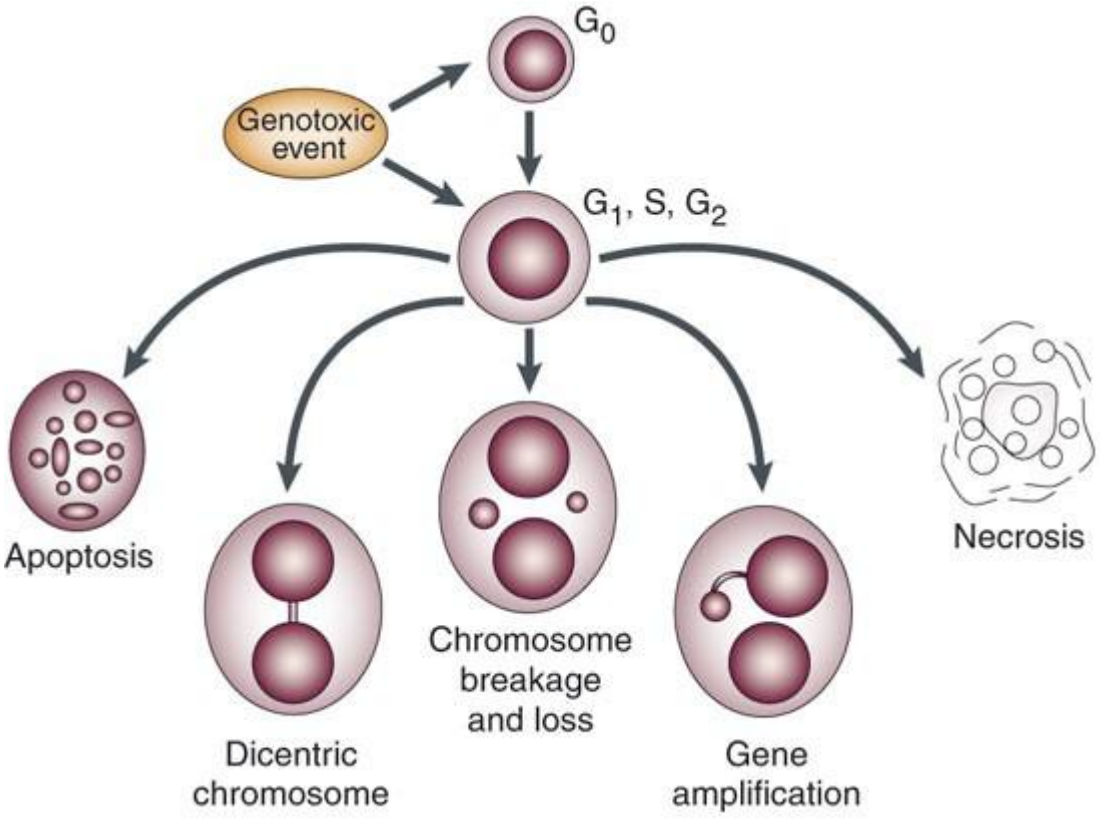

Figure 6. Using these biomarkers within the CBMN assay, it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement, for example, dicentric chromosomes (NPB), gene amplification (NBUDs), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of mono-, bi- and multinucleated cells (Adopted by Fenech 2007)

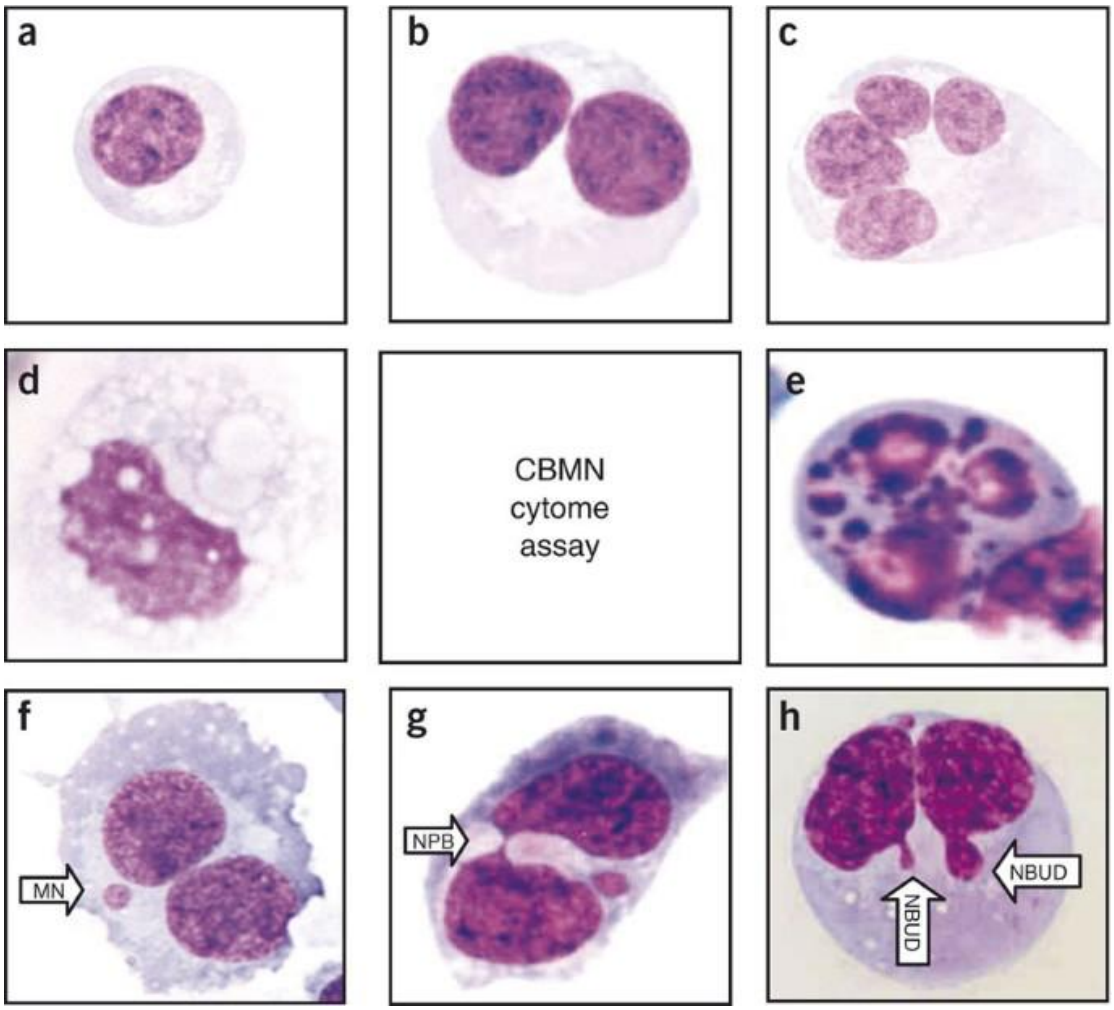

Figure 7. (a) Mononucleated cell; (b) BN cell; (c) multinucleated cell; (d) early necrotic cell; (e) late apoptotic cell; (f) BN cell containing one or more MNi; (g) BN containing an NPB (and a MN); (h) BN cell containing NBUDs. The ratios of mononucleated, BN, multinucleated, necrotic and apoptotic cells are used to determine mitotic division rate or NDI (a measure of cytostasis) and cell death (cytotoxicity). The frequency of BN cells with MNi, NPBs or NBUDs provides a measure of genome damage and/or chromosomal instability. (Adopted by Fenech 2007)

1.8.3 Cytokinesis-block proliferation index (CBPI) The overall cytotoxicity of an agent in a cell culture results in both cytostasis and cell death. Cytostasis is not restricted to inhibitors of cell division but may also be the consequence of many cytotoxicity pathways leading to a delayed cell cycle. As cytostasis may result from effects on cell division and may also be cell death-related, this component of cytotoxicity could be predominant and therefore should be accurately taken into account in the overall cytotoxicity. In the in vitro micronucleus test, when using cytochalasin B, two main methods for measurement of cytotoxicity have been recommended. One is based on CBPI (Cytokinesis-Block Proliferation Index), where CBPI is determined in treated (CBPIT) and control (CBPIC) cultures (159). The cytokinesis block proliferation index (CBPI) is given by the following equation:

$$\text{CBPI} = \frac{\boldsymbol{M_1 + 2M_2 + 3(M_3 + M_4)}}{\mathbf{N}}$$

where M1, M2, M3 and M4 correspond to the number of cells with one, two, three, and four nuclei respectively and 'N' is the total number of cells. For CBPI calculation, 2,000 cells were counted. CBPI is a tool that is used in order to better understand BNMN results from cell cultures where cytochalasin B is used. Moreover, it is able to provide substantial information regarding possible cytotoxic effects (necrosis, apoptosis or cytostasis) on the cell culture induced by any chemical agents. If CBPI remains close to the numeric value of one, then there is no cytotoxic event. Moreover, should it remain almost the same between time-points, then MNf results are comparable and any fluctuation of MNf can be attributed solely to the parameter of interest (in our case CRC and/or the systemic treatment). These parameters were calculated, in order to determine the possible cytotoxic effects induced by either the chemotherapeutic agents, the cancer itself or any other cytotoxic agent.

1.8.4 Scoring criteria for the CBMN assay using isolated human lymphocyte The cytokinesis-blocked cells that may be scored for MN frequency should have the following characteristics (Figure 8) (160)**:**

a) The cells should be binucleated
b) The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary
c) The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity
d) The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter.
e) The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable
f) The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells

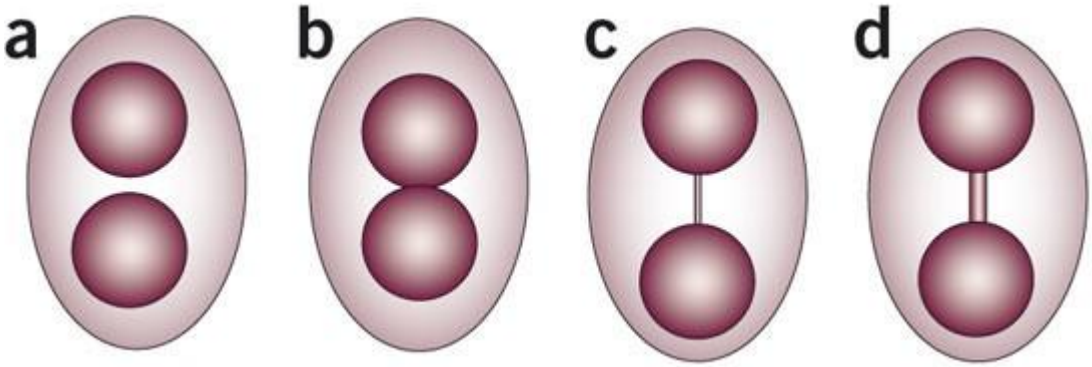

Figure 8. Criteria for choosing binucleate cells in the cytokinesis-block MN assay: (a) Ideal binucleate cell; (b) binucleate cell with touching nuclei; (c) binucleate cell with narrow NPB between nuclei; (d) binucleate cell with relatively wide NPB. Cells with two overlapping nuclei may be considered suitable to score as BN cells if the nuclear boundaries are distinguishable. Occasionally, BN cells with more than one NPB are observed (Adopted by Fenech 2007)

MN are morphologically identical to but smaller than the main nuclei. They also have the following characteristics (Figure 9, 10):

a) The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.
b) MN are round or oval in shape
c) MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles
d) MN are not linked or connected to the main nuclei
e) MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary
f) MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense

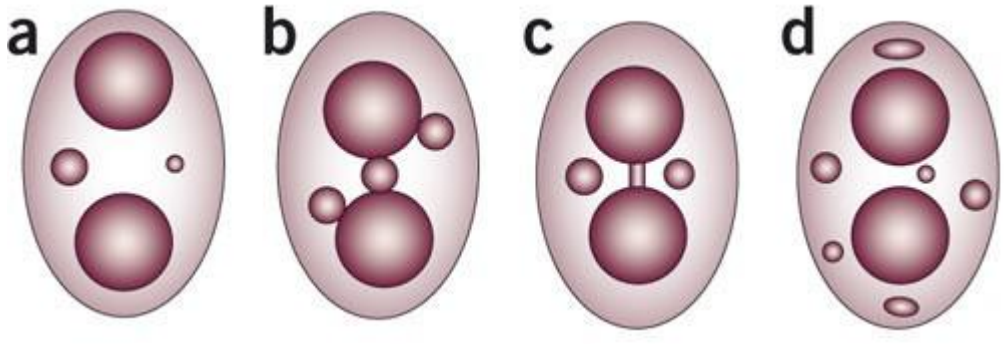

Figure 9. Typical appearance and relative size of MN in BN cells: (a) Cell with two MN one with 1/3 and the other 1/9 the diameter of one of the main nuclei within the cell. (b) MN touching but not overlapping the main nuclei. (c) A BN cell with NPB between main nuclei and two MN. (d) A BN cell with six MN of various sizes; this type of cell is rarely seen in cells that are not exposed to high doses of genotoxins (Adopted by Fenech 2007)

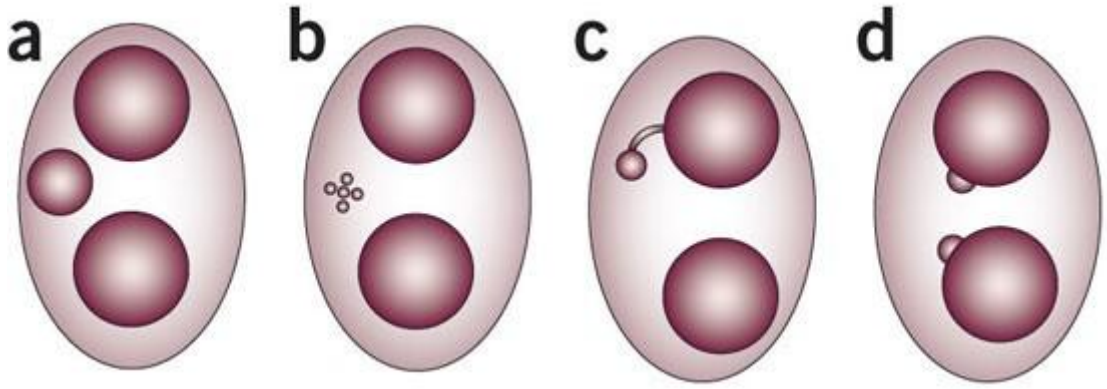

Figure 10. BN cells (or cells that resemble BN cells) that should not be scored as MN originating from chromosome loss or chromosome breakage: These situations include (a) a trinucleated cell in which one of the nuclei is relatively small but has a diameter greater than 1/3 the diameter of the other nuclei; (b) dense stippling in a specific region of the cytoplasm; (c) NBUD that appears like an MN with a narrow nucleoplasmic connection to the main nucleus and (d) nuclear blebs consisting of nuclear material protruding from the nucleus but without an obvious constriction or bridge between the protruding nuclear material and nucleus (Adopted by Fenech 2007)

Criteria for scoring apoptotic cells:

a) Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries
b) Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane
c) Staining intensity in the nucleus, nuclear fragments and cytoplasm is usually greater than in viable cells

Criteria for necrotic cells:

a) Early necrotic cells can be identified by the presence of a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus
b) Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary
c) Staining intensity of the nucleus and cytoplasm is usually less than that observed in viable cells

1.8.5 Methylation status, CpG islands and Micronuclei formation Epigenetics has recently become a very promising target for manipulation in molecular biology because of the growing evidence of its involvement in chromatin status regulation, gene expression; and both epigenetics and genetics have an equal influence on the development of genomic instability and cancer. The greatest potential of epigenetic alterations is their reversible nature in contrast to mutations which made epigenetics so attractive for therapeutic research (161). A similar correlation between MN induction and DNA hypomethylation was shown in radiation-induced bystander cells. An increase in the level of MN, DNA DSBs, and apoptosis was parallel to the loss of nuclear DNA methylation in bystander human cells after microbeam radiation. Analysis of the fate of MN in the cells has recently regained a lot of interest. The recent study by Utani et al. suggested that MN formed after mitosis were stably maintained in the cells for up to one cell cycle. Furthermore, mitotic division of cells with MN led to formation of daughter cells either with or without MN (162). The ability of MN DNA to replicate itself remains obscure, but some suggestions have been made that MN replication depends on MN nature, and if it happens, usually it occurs at the same time as main nucleus replication. Similarly, MN transcription events depend mainly on MN structure. MN containing whole chromosomes showed active transcription, whereas acentric fragments were not able to synthesize RNA, unless they represented transcriptionally competent DMs (163). It should be emphasized here that any possible transcriptional activity in MN depends on nuclear envelope integrity and the presence of nuclear pore complexes. According to Fenech et al. (164), the main mechanism of MN formation originated from chromosome malsegregation is hypomethylation of centromeric and paracentromeric regions – satellite repeats. Usually, satellites are hypermethylated, and loss of methylation elongates repeat regions decreasing the tension in kinetochores and thus creating wrong connections between microtubules of the mitotic spindle and chromosomes. The role of DNA methylation is crucial for normal development, proliferation, and genome stability. The distribution of CpG-dinucleotides is not random in the genome. Most of CpG sites are clustered in promoter areas of genes creating so-called CpG

islands. Usually, promoters of tumor suppressor genes are hypomethylated to allow their expression for normal functioning of cells, whereas oncogenes and some repeat elements are silenced through hypermethylation, thus maintaining genome integrity. Reanimated transposons can lead to translocations, gene disruption, and chromosomal instability. X chromosome inactivation is also a result of hypermethylation. Centromeric regions of chromosomes are heterochromatic and lay within tandemly repeated DNA. Constitutive heterochromatin of centromeres is epigenetically silenced by histone methylation (H3K9Me3 and H3K27Me) and DNA hypermethylation, thus enabling a low frequency of recombination and the repression of transcription. However, undermethylation of repeated DNA sequences and satellite DNA in the centromeric and pericentromeric regions of chromosomes is highly linked to karyotypic instability found in a variety of cancers. The possibility exists that DNA hypomethylation in the centromeric region may modify a platform for the correct kinetochore orientation and attachment to the spindle, resulting in improper chromosome segregation and MN formation. Such hypothesis is mainly supported by ongoing experiments involving DNA methylation activators/inhibitors which affect MN formation.

1.8.6 Genotoxicity and Micronuclei Understanding of the mechanisms of MN formation induced by genotoxic agents is of a great significance for both the detection of diseases such as cancer and their treatment. The manipulation of such mechanisms may be beneficial for both the prevention of MN formation and development of diseases and the induction of MN for therapeutic purposes. In fact, it is a matter of choosing the right target in the process of MN formation. Epigenetic regulation of MN formation includes at least four outlined mechanisms: a) DNA methylation, b) histone modifications, c) chromatin remodeling, and d) non-coding RNA expression. Nonetheless, their presence in a healthy individual is not unusual although they tend to be more common among people adhering to unhealthy lifestyles. Generally, younger individuals have lower MNf than the older individuals. In detail, an age-related increase in chromosome damages and MN formation in lymphocytes has been described. Analysis of population data from 12 Italian laboratories in the mid 1980s–1990s showed the most dramatic increase in MN in the age group of 50–59 that remained unchanged thereafter (165). The age-associated incline in CA and MN may be caused by a decline in DNA repair and the aneuploidy phenomenon. Genomic instability and oncogenicity cause the accumulation of DNA damage with age. Oxidative damage can also contribute to MN frequency during ageing. The baseline MN frequency in newborns and children is relatively low, but higher susceptibility to DNA damages in children may rapidly increase the MN formation due to environmental exposure to genotoxic agents. Gender factors have been studied in parallel with aging. Mainly, a higher MN frequency has been reported for women. Similarly, the effect of gender was described for MN associated with aneuploidy (centromere-positive MN), which was higher in females. On the other hand, MN testing is widely used for the evaluation of genotoxicity of different anti-cancer drugs. Adriamycin is an anthracycline drug with strong mutagenic properties that increases MN incidence up to 10- to 15-fold and significantly declines cell survival. Curcumin alone induces MN in PC12 cells but reduces the total frequency of MN induced by cisplatin, thus showing both genotoxic and antigenotoxic properties, depending on prescription protocols. Similarly, anti-cancer drugs, gemcitabine and topotecan, increase abnormal metaphases and the number of MN in mouse bone marrow. The

CBMN assay showed the stimulation of DNA damages in V79 Chinese hamster cells after combinational treatment with bleomycin and DNA-PK inhibitor wortmannin. Interestingly, given that genotoxicity is linked to chromosome aberrations, it is expected that cigarette smoking would cause MN. Surprisingly, most studies deny the ability of smoking compounds to induce MN. In the Human MicroNucleus project, 1409 current smokers and 800 former smokers were tested for MN in lymphocytes. Both groups showed a decrease in MN frequency compared to non-smokers (166). Although, when tobacco-specific nitrosamine (NNK) was added to the culture of the repair-deficient fibroblasts, the frequency of MN was doubled suggesting that smoking could induce MN in repair-deficient cells. Finally, numerous studies have shown that MNf is a sensitive biomarker of various types of cancers such as lung, bladder and CRC, suggesting that cancer patients exhibit higher MNf than healthy individuals (167). Although a number of studies has explored MNf in CRC, it is not clear what the course of MNf is in the long-term, and especially how MNf is correlated with prognosis.

1.8.7 Correlation between Micronuclei and Cancer MN are one of the four main endpoints, together with chromosomal aberrations, aneuploidy, and sister chromatid exchange (SCE) in the identification of cancer initiation. A large number of papers describe the correlation between MN and cancer development. A significant increase in MN in lymphocytes was shown in untreated cancer. Furthermore, healthy women with BRCA1 and BRCA2 mutations showed a higher increase in MN frequency and a higher radiation sensitivity than women without family history of breast cancer. Similar outcomes were shown in lung cancer patients with a high frequency of spontaneous MN, as well as in patients with pleural malignant mesothelioma, and adenocarcinoma patients. Cancer-prone patients with Bloom syndrome and ataxia telangiectasia also possess a high frequency of MN in lymphocytes. Analysis of European cohorts indicates that individuals with increased MN are more likely to get cancer 12–15 years after the test was performed (168).

1.8.8 Micronuclei and CRC A growing body of evidence indicates that the loss of genomic stability is a key molecular pathological step that occurs early in carcinogenesis. Chromosomal instability is the most common type of genomic instability in CRC and it occurs in 80–85% of colorectal tumors. The genome of colon cancer is often marked by chromosome rearrangement, alterations in chromosome number (aneuploidy) and gene amplification. These events may represent promising biomarkers for the prevention and risk assessment of CRC. Scientists have demonstrated that the level of genetic damage in peripheral blood lymphocytes (PBL) reflects the amount of damage in the precursor cells, which subsequently leads to the carcinogenic process in target tissues. The use of biomarkers associated with this event may provide effective tools for the early detection of the changes related to cancer. Over the last decade, micronucleus (MN) analysis in PBL has been proposed as a useful biomarker for this purpose (169). Micronuclei originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. The formation of micronuclei during the dividing process can be caused by chromosomal rearrangements, altered genome expression or aneuploidy, all of which are associated with the chromosome instability phenotype, often observed in cancer patients (170). The hypothesis of an association between MN frequency and cancer development is supported by a number of observations, the most substantial of which include the high MN frequency in untreated cancer patients and in subjects

affected by cancer-prone congenital diseases and a number of international cohort studies, which have demonstrated that the MN frequency in the PBL of healthy subjects is a predictor of cancer risk. It is widely accepted that chronic inflammation can contribute to colon carcinogenesis by producing oxidative stress, which is defined as over-production of oxygen species combined with the insufficient protective mechanism of antioxidative defense. The increased production of superoxide radicals may induce the release of chromosome-damaging material, the so-called clastogenic factors (CFs), in circulating plasma. These low-molecular weight substances (lipid peroxidation products, inosine nucleotides and cytokines) are produced via superoxide stimulation in a multistep process that increases the production of superoxide radicals perpetuating and enhancing chromosomal damage. MN frequency was significantly higher in the CRC group than it was in the polyp and control groups. Moreover, increased MN frequency, even if lower than that detected in CRC patients, was identified in the lymphocytes of the adenoma polyp group with respect to controls. Furthermore, the statistical analysis that was conducted using the Poisson model indicated that the RR of the increase of MN frequency was associated with the pathological status of the subjects. This suggests that the MN frequency varies following the CRC dynamics through the different main stages of this pathology. It is also demonstrated that ~40% of the subjects that tested positive in the FOB test had no histological lesion linked to colon carcinogenesis, showing that the FOB test can produce a high rate of false positive results (171). Furthermore, Karaman et al. exhibited increased MN frequencies in the lymphocytes of CRC and colon polyp patients, could support these observations, as the induction of changes in DNA that lead to mutations plays a role in carcinogenicity (172).

1.8.9 Micronuclei assay It is well documented that micronuclei frequency represents a sensitive indicator of genetic damage induced by various xenobotics and environmental stressors. Among the various methods used for evaluating genetic damage, the most common are: a) molecular (PCR and agarose gel electrophoresis, quantitative PCR, ligation-mediated PCR, immuno-coupled PCR), b) fluorescence [sister chromatid exchange assay, chromosomal aberrations assay, Comet assay, alkaline single-cell gel electrophoresis, neutral single-cell gel electrophoresis, lesion-specific enzymes assay, bromodeoxyuridine-labelled DNA-comet FISH, halo assay, Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay, DNA breakage detection (DBD)-FISH, FCM-Annexin V labeling, Radioimmunoassay (RIA)], c) chemiluminescence (ELISA, Immunohistochemical assay , Immunological assay) and d) micronucleus assay. For the needs of this doctoral thesis, the micronucleus assay was chosen on the basis of two main advantages: a) The relatively easy evaluation of the parameter in concern and b) the greater statistic power due to the higher number of counted cells instead with the metaphase analysis assays.

1.9 Telomerase

1.9.1 Telomerase function and regulation Telomeres are repetitive DNA sequences (TTAGGG) located at the end of chromosomes which are associated with the Shelterin complex. Among its main functions, telomeres protect chromosomes from end-to-end fusion and DNA degradation. The end replication problem, owing to the inability of DNA polymerase to replicate the 3’ end of chromosomes, causes a progressive telomere shortening which is coupled to cell division. When a critical

telomere length is reached, cells activate the protective mechanisms, i.e. senescence & apoptosis, in order to avoid the proliferation of genetically unstable cells. If these mechanisms fail, cells continue to divide and enter into a crisis stage which is characterized by massive genomic instability and cell death. Telomerase is a ribonucleoprotein enzyme complex, consisting of two subunits; hTERC (human telomerase RNA component), an RNA sequence pattern on which the synthesis of telomeric parts is based, and a protein called human telomerase reverse transcriptase (hTERT), whose responsible gene is located in chromosome 5 (5p15.33) (Figure 11).

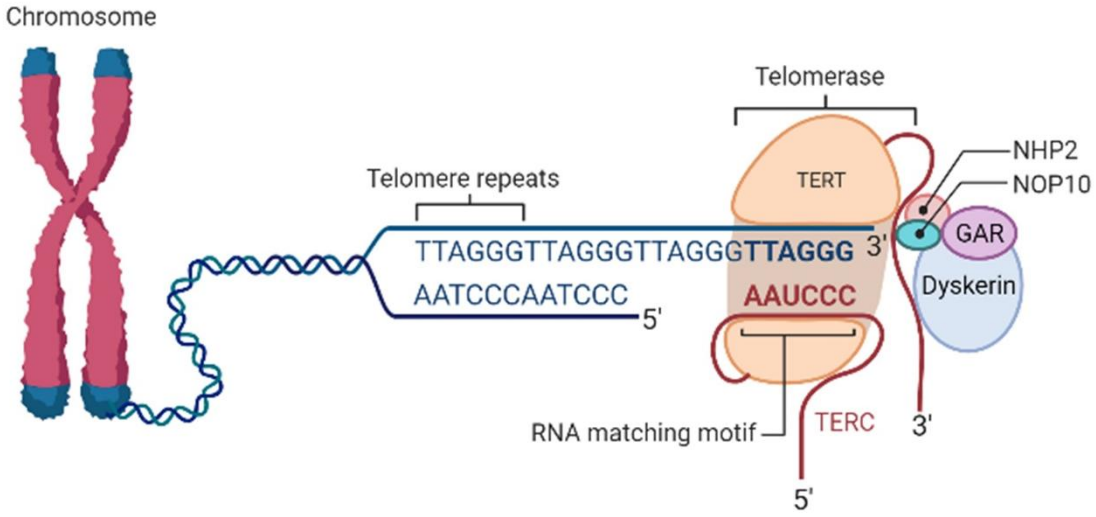

Figure 11. Illustration of the telomerase complex. Created with BioRender.com

Two of the six subunits of shelterin, TPP1 (tripeptyl peptidase I) and POT1 (protection of telomeres 1), regulate the action of telomerase. hTERT is responsible for the suppression of apoptosis in an early phase before the induction of cytochrome c. Normally, TA is elevated in the early gestation period. hTERC is broadly distributed in the tissues, both present in normal and cancerous cells, while hTERT expression occurs mainly in germ-line cells and most tumor cells. hTERT is required for the unrestricted cellular growth and as a result it plays a leading role in tumor initiation and further progression. Telomerase, however, requires also additional enzymes and proteins for its stability and proper function. Dyskerin, functioning as RNA binding protein and enzyme, is a pseudouridine synthetase. Although this function is not of importance for telomerase, it is shown that its presence is obligatory for the enzyme. Moreover, TCBA1 (telomerase Cajal body protein 1) is associated with telomerase activity through dyskerin. Modulation of telomerase activity occurs in several biological tiers; transcription, mRNA splicing, sub- cellular localization of each component and the assemblage of TR and hTERT in an active ribo-nucleoprotein complex. Transcription of hTERT gene is most likely the key for the modulation of telomerase activity. The hTERT gene has a length of 35 kb, entailing 16 exons and 15 introns. For the total activation of hTERT promoter, the concurrent action of MYC and SP1 is deemed essential. TP53, when coming in interplay with SP1, downregulates TERT. Nuclear factor-kB, hypoxia-inducible factor (HIF-1) and the ETS/MYC complex are also involved in the positive regulation of hTERT promoter expression. Moreover, the histone methyltransferase SMYD3 incites TERT expression in healthy as well as malignant cancerous cells. Expression of TERT is constricted by the onco-suppressor genes WT127 and MEN1, and also via

MAD/MYC and TGF-β/ SMAD pathways. TERT expression is also suppressed by the inhibitors p16INK4a and p27KIP1. Post-transcriptionally, regulation of telomerase may take place, mainly, via tissue-specific alternative splicing mechanisms. Notwithstanding that, the telomeres' length is induced in each cell cycle in a lower rate, endorsing cellular senescence (173). Loss of DNA sequences occurs mainly due to two parameters: the end-replication problem, that is the dependence of DNA polymerase on promoters (3' telomeric ends) that undergo degeneration and suppression of telomerase. At this point, the activation of telomerase compensates for telomere shortening and allows cells successfully exit from the crisis stage. Telomerase is specifically expressed in immortal cells, such as stem cells, germ tissues and cancer cells. For the latter, telomerase confers an unlimited replicative potential and has been implicated in immortalization and carcinogenesis (Figure 12).

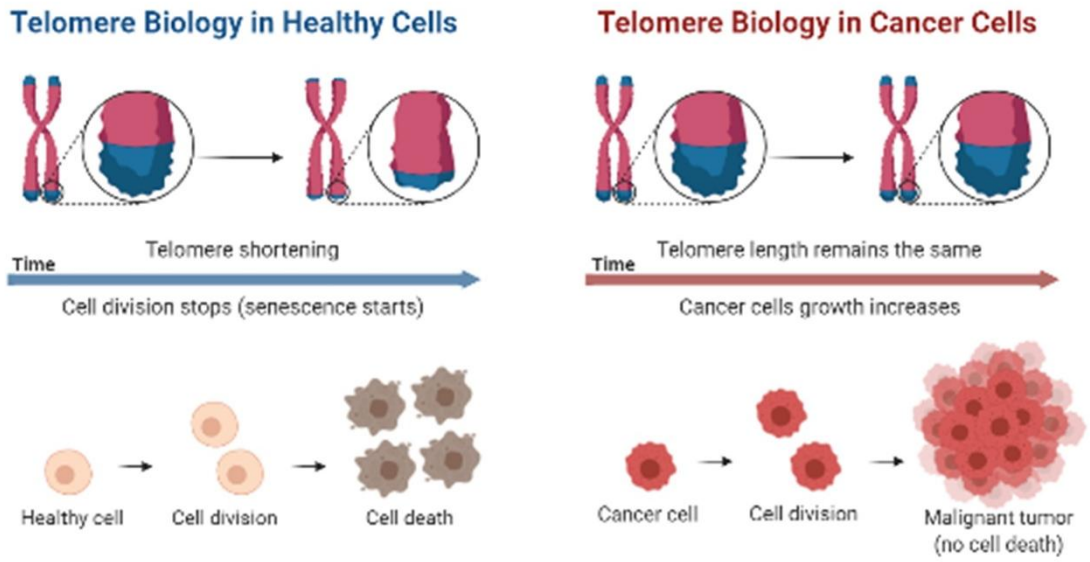

Figure 12. Illustration of the differential mechanism of chromosome attrition in normal and cancer cells. Created with BioRender.com

Telomeres with critical length incite checkpoint signalling mechanisms found in the p53 pathway. The p53 tumour-suppressor downregulates hTERT gene. It was observed that a mutant form of TERT presents the same antiapoptotic activity and that p53-mediates downregulation of hTERT, which is critical for efficient p53-dependent apoptosis. The uncapping of telomeres, instigated by the disruption of TRF2, induces p53 as it represents a signal for breakage (through various mediators, like ataxia-telangiectasia mutated kinase (ATM kinase)). In case protective mechanisms are inactivated, e.g. that of TP53 protein, cells continues with proliferation. By this way, further corrosion of telomeres incites functional impairment regarding telomeric end protection, thus leading to chromosomal instability. Consequently, the erosion of telomeres may act as following; tumor suppression due to induction of senescence, and simultaneously tumor promotion by provoking genomic instability. Brief telomeres could also induce genome-wide DNA methylation, an event that can regulate oncogene and onco-suppressor gene expression. Overall, the telomere hypothesis supports the notion that shortening of telomeres to a critical length fosters cells to evade further division, whereas protection of telomeres favors proliferation. When the action of telomerase is inhibited, the result is cellular senescence. On the contrary, expression of telomerase in ectopic sites permits cellular division, as the length of telomeres remains intact (174,175).

1.9.2 Telomerase independent elongation Some cancers are telomerase negative and their telomere lengths are maintained by a mechanism known as alternative lengthening of telomeres (ALT). This alternative mechanism (ALT), involves the synthesis of new telomeric DNA from a DNA template via homologous recombination (HR), in contrast to the telomerase dependent elongation. ALT cells can be classified in four different groups according to the recombination mechanism that they use. In human cancer cells where ALT is up-regulated, a characteristic telomere phenotype is detected. ALT cells present heterogeneous telomere lengths and longer telomeres than cancer cells which maintain the telomere status using telomerase. The ALT mechanism is used less often than telomerase. ALT cells include the promyelocytic leukemia (PML) nuclear bodies that contain telomeric DNA and telomeric binding proteins and other proteins involved in the recombination process. These ALT-associated PML bodies (APBs) may be used to identify cells which use the ALT pathway. ALT is characteristic of some tumors such as neuroepitelial tumors, osteosarcomas, in some types of soft tissue sarcomas and occurs in low frequency in some types of carcinomas. In colon carcinogenesis, we suggest this alternative mechanism to maintain TL in case that TA was negative (176,177).

1.9.3 Telomerase activity and CRC Approximately 85% of CRCs are characterized by chromosomal instability and telomere dysfunction may be considered a major driving force in generating this feature. Telomere shortening is an early event in CRC carcinogenesis and telomere/telomerase dysfunction is considered as a fundamental player in this process. For telomerase, there is a general consensus that high hTERT levels or telomerase activity are associated with poor prognosis. In non-cancerous somatic cells, TA is undetectable or present at low levels. Cellular senescence is a key barrier against cancer, which implies that cancer cells have been transformed to immortal cells. This fact requires increased levels of TA, in order not to decrease telomere's length. This mechanism is explained by the hTERT promoter, whose upregulated expression is promoted by differential hTERT gene expression in neoplastic and normal cells. For example, Chen et al demonstrated that a net increase of hTERT units is possible through upregulation of SPT5, a tumor-specific hTERT promoter-binding protein encoded by the upregulated SUPT5H gene (178), while Ling Zhang et al using the HCT-116 cell line (a KRAS mutated line), exhibited increased TA via upregulation of the T-STAR gene (which encodes a number of proteins responsible for multiple functions in pre-mRNA splicing, signaling and cell cycle control) (179). Given the implication of telomeres' length in CRC, TA has attracted scientific interest as well. Jian Zou et al identified that telomerase is found to be activated in 90% of malignant tumors (180). Interestingly, TA has been detected in early stages of CRC which would mean that it is a determining factor during carcinogenesis (181) while increased hTERT expression and elevated plasma concentration of circulating TERT mRNA have also been identified as an unfavorable independent prognostic marker of overall survival in patients with stage II CRC (182). However, the prognostic role of telomere length in CRCs still needs to be confirmed. Recently, the clinical utility of telomeres has been demonstrated to improve the treatment of metastatic colorectal cancer: the outcome of patients treated with anti-EGFR therapy seems to be dependent on the tumor telomere length. While most studies identify telomere shortening as a critical initial event in carcinogenesis, the

role of telomere length in cancer cells as a marker of disease progression is controversial. In fact, considering CRC, mean telomere attrition has been associated with the lymph node invasiveness of colon cancer cells, whereas other works indicated that telomere length in cancer tissue was significantly longer in the late stage of colorectal tumors.

1.9.4 Telomerase activity as diagnostic and prognostic marker in CRC Kim et al. was the first group which studied the TA by means of the TRAP (telomeric repeat amplification protocol) method. These authors determined that 90 % of tumors had TA but not the normal cells. They also believed that TA may be a prognostic and diagnostic factor in the future (183). The simplicity and sensitivity of the TRAP method were quickly improved. As a result, the levels of TA can be measured and not only the positive/negative classification, as happened at the beginning. These improvements in the technique allow determining the low levels of TA in the normal mucosa. The authors attribute this TA in the normal mucosa as micrometastasis of the tumor in the adjacent mucosa. However, it can be due to the presence of the proliferative cells, which are located in the intestinal crypts, or lymphocytes. TA in CRC was detected in 80–100 % of tumor samples and telomerase activation is a universal step in the carcinogenesis process, whereas TA was detected in 9–53 % in normal mucosa. In the vast majority of studies, tumor mucosa presents a higher TA than the corresponding normal mucosa. In this situation, where the TA in normal mucosa is not null, it would be a good value to use the telomere index (TI) which results from this formula: log (TA tumoral mucosa–normal mucosa). TI is the net TA in the tumoral mucosa. The use of telomerase as a diagnostic biomarker in CRC has been considered using the novel assay Telomerase Biosensor Technology (TBT; Sienna Cancer Diagnostics, Melbourne, Australia), which is predicted to allow 95 % specificity (few false positives) and 95 % sensitivity (few false negatives) for bladder cancer and melanoma, as well as CRC. Some authors have found that TA is an independent prognostic marker of recurrence, disease-free and overall survival in patients with CRC and there is a general consensus that high levels of TERT and/or TA are associated with poor prognosis in CRC. TI is described to be an independent prognostic factor for predicting the recurrence and overall survival in the first 2 years after surgery in rectal cancer but not in patients with colon cancer. This fact suggests a different telomerase behavior depending of the tumor localization. These results are according with other studies which identified differences in the etiology, pathologic and clinical behavior of colon and rectal cancers. Local recurrence is more frequent in rectal cancers patients and distant metastases are more frequent in colon cancers patients. So it is reasonable to suggest different etiologic factors, molecular basis, diagnostic and prognostic factors between colon and rectal cancer. These results support the idea that the large bowel cannot be considered a uniform organ, at least for cancer biology. Tatsumoto et al. classified patients with CRC according to high TA (>50) and low TA (<50) and they found significant differences between recurrence-free survival and overall survival. Patients with higher TA had worse clinical prognosis (184). In contrast, the worst prognosis was found for patients with telomerase-negative tumors, according to the results of Kawanishi-Tabata et al. These authors only considered stage II CRC patients and this could be the explanation of this disparity (185). In the years following, Sanz-Casla et al. demonstrated that patients with TA in the tumor showed a higher risk of death or recurrence, independently of other factors with prognostic value such as tumor stage and tumor location (186).

Moreover, others research articles proved the usefulness of TA determination to predict the recurrence and survival in the CRC patients operated on. An indirect way to study TA is by determining the hTERT expression, as the acquisition of hTERT expression seems to be an essential step for the TA in the majority of the human tumors. There is a strong correlation between the hTERT expression and TA, and in the latest years a correlation with the hypermethylation of the hTERT promoter, in different cancer types included CRC, has been described. Methylation of the hTERT promoter is necessary to activate its transcription (methylated promoters are usually related with silent transcription, but this is an exception). Bertorelle et al. found that hTERT level is an independent prognostic marker of overall survival in CRC patients (187). Moreover, the identification of TERT transcripts in the plasma of CRC patients could be a useful tool to monitor the disease, as the levels of TERT mRNA are correlated to those in tumors. In summary, there is sufficient data to consider telomerase as useful marker for monitoring and predicting the CRC outcome. The telomerase analysis, to predict the clinical prognosis of CRC patients, could contribute to the identification of groups of patients susceptible to receiving therapies aimed at the inhibition of telomerase. It was reported that TA is strongly associated with CRC, in terms of incidence, progression and metastatic activity. Moreover, an increased level of hTERT expression and TA was often observed in rectum and left-sided adenocarcinomas, in comparison to those of the right colon. Moreover, colon cancers entailed more telomerase activity than rectal cancers. An important finding was the eliminated telomerase activity in patients with Dukes C or D stage in comparison to patients with stage A or B, indicating that TA occurs at early stages of disease (175).

1.9.5 Methods for TA estimation The most common methods for detecting telomerase activity are TRAPs (telomeric repeat amplification protocols), which allow one to perform semi-quantitative and quantitative analyses, using some of their modifications. Such modifications include: increase of the analysis rate, replacement of the radioactive label by nonlabeled compounds, the reduction of the amount of side products, etc. Among these methods are the scintillation proximity assay, hybridization protection assay, transcription amplification assay, and the magnetic bead-based extraction assay. Some modifications even enable to detect telomerase activity within a single cell. The telomeric repeat amplification protocol can be subdivided into three main stages: primer elongation, amplification of telomerase-synthesized DNA, and thirdly, its detection. At the elongation stage, telomeric repeats are added to the telomere-imitating oligonucleotide by telomerase present in the cell extract. Then, PCR-amplification of telomerase-synthesized DNA is carried out with the use of specific primers (telomere-imitating and reverse primers). At this stage, different labels can be incorporated into the telomerase-synthesized DNA, such as radioactive, fluorescent, or affine labels. This stage is then followed by detection (in the original method, it comprises electrophoretic separation of PCR products and photographing). The original TRAP method has several drawbacks. Initially, the CX oligonucleotide, which complementarily overlaps with TS for several base pairs, is used in the amplification of PCR products. It results in the dimerization of primers and products that emerge due to the interaction between primers. Even when using the optimal ACX primer with the noncomplementary TS end, a background signal may emerge during the analysis of concentrated extracts from tumor tissues. An additional problem is encountered when using reverse primers which completely correspond to

telomeric repeats. The primers are not annealed at the matrix edges during PCR (as a result of periodicity in telomeres), and hence false signals emerge. This problem is typically solved by adding regions that are noncomplementary to telomeres with a 5'-end nontelomeric "appendix" made of 6 bp to the primer ends. In order to reduce nonspecific signals, it is possible to use a combination of several primers that are used as reversed ones. Oligonucleotide TSG4 can also be added to the TRAP mixture in order to assess the effect of duplex-stabilizing inhibitors; this oligonucleotide does not require the synthesis of several repeats by telomerase before the inhibitor begins its action. Moreover, in the case when PCR is used for signal amplification, the PCR inhibitors contained in the specimen can impact the results of telomerase activity detection. Originally, in the TRAP method, PCR products were detected in polyacrylamide gel (PAAG) on account of the radioactive label, which was introduced using a radioactively labeled primer or incorporated into the DNA during the reaction. The method allows to perform a qualitative assessment of the activity and processivity of telomerase in cell and tissue extracts; however, as previously stated this requires radioactive specimens. PCR at the second stage of TRAP allows to obtain an amount of DNA sufficient for gel staining, e.g., using ethidium bromide (an appreciably strong mutagen with a low sensitivity), silver nitrite (its sensitivity is equal to that when using the radioactive label; however, the method is more laborious and relatively expensive), and Sybr Green and its analogs (its sensitivity is equal to that when using the radioactive label, while mutagenicity is considerably higher than that in ethidium bromide, although it is an intercalating dye, as well). Fluorescent labeling of the nucleotides employed in TRAP can also be used. Over the years several versions of the TRAP method have emerged: a) TRAP with an additional specific reverse primer ("two-primer" TRAP). Two-primer TRAP is a modification of the standard TRAP which is used to reduce false signals), b) TRAP with fluorescence resonance energy transfer (FRET). One of the alternatives to DNA staining in the TRAP method is to use primers with energy transfer (amplifluors). The method involving the use of the time-resolution fluorescence resonance energy transfer (HTRF) combines the standard FRET technique and a long-lived fluorescent donor. The method is based on the use of europium or terbium cryptate complexes, c) TRAP with detection using the scintillation proximity assay. Another means for the detection of the DNA amplified in TRAP without PAAG is the scintillation proximity assay, d) TRAP with detection using the hybridization protection assay. TRAP modification in which the hybridization protection assay (Hybridization protection assay-TRAP) is used is a safer modification, e) TRAP combined with enzyme-linked immunosorbent assay (ELISA). In the TRAP-ELISA method, DNA after the amplification is determined colorimetrically, facilitating the qualitative and semi-quantitative assessment of telomerase activity, f) TRAP with electrochemical detection. After the PCR, the nonreacted nucleotide triphosphates can be separated, followed by treatment of the remaining products with 3 M HCl, g) TRAP with real-time PCR. Real-time PCR is used for simultaneous DNA amplification and measurement of the amount of products obtained after each amplification cycle. Standard TRAP, combined with real-time PCR, allows to obtain quantitative results, h) TRAP on microchips. TRAP on microchips is the combination of the two-primer TRAP and binding of PCR products on chips, followed by probe hybridization and detection. Other than the various versions of the TRAP method, methods for TA measurement also include TERT mRNA by competitive polymerase chain reaction (PCR) and telomerase activity by TRAP assay, TERT mRNA by real-time PCR, TA by TRAP assay-based

enzyme linked immunosorbent assay (ELISA) and by TRAP assay-based immuno-fluorescence assay. The method of qRT-PCR (quantitative real-time polymerase chain reaction) is a tool for detecting median telomere length, for the assessment of the cellular response to ageing. For the assessment of telomerase levels, two main aspects are targeted: quantification of hTERT mRNA levels and quantification of telomerase activity. To achieve that, PCR could be utilized. The most effective way is to create primers that have the ability to bind to the a and β sites and by this way identify the overall mRNA, responsible for encoding the functional protein product. In the frame of telomerase state in carcinogenesis, in situ hybridization performed in hTERT gene is optimal, compared to TRAP TA and RT-PCR. However, telomerase is highly expressed in stem and cancer cells. When telomeres are shortened, senescence and ageing mean reduced mobilization of stem cells. When telomeres are overexpressed, similarly the mobilization of stem cells is increased, meaning that this deviant stem cell mobilization might contribute to carcinogenesis and genetic mutations. Telomeres in CRC are briefer than in the adjacent normal mucosa. Nevertheless, multiple studies agree upon the role of telomerase as a marker of colorectal carcinogenesis. Due to the existence of intestinal crypt basal cells, the healthy mucosal part may present slight hTERT mRNA and telomerase activity (188,189).

1.9.6 TRAP combined with enzyme-linked immunosorbent assay (ELISA) In the TRAP-ELISA method, DNA after the amplification is determined colorimetrically, facilitating the qualitative and semi-quantitative assessment of telomerase activity. Biotin-conjugation of the TS primer allows for the binding of the amplified DNA to streptavidin-coated microplates. The amplified DNA denatures and is hybridized with digoxigenin (DIG)-labeled probes, which demonstrate specificity towards telomeric repeats, and binds to the microplates due to the streptavidin–biotin interaction. This complex can be detected using polyclonal sheep antibodies to DIG conjugated to horseradish peroxidase; the activity of the latter is determined colorimetrically. This method differs from the hybridization version by the emergence of a second step of signal amplification due to the enzymatic reaction. One of the drawbacks of the TRAP-ELISA method is the complexity in separating the telomerase-positive and telomerase-negative controls, which may result from the absence of internal controls and two steps of signal amplification. Nevertheless, the TRAP-ELISA method is faster as compared with TRAP, which is based on the separation of the amplified DNA in gel. This fact makes it possible to use the TRAP-ELISA method in screening studies. The method is suitable for semi-quantitative determination of telomerase activity in tissue and cell line extracts. The sensitivity threshold of this method is the 10-cell extract of the telomerase-positive cell line.

3. Aim of this study

Excluding skin cancer, colorectal cancer is the third most prevalent and lethal cancer among both men and women in the United States. It was estimated that in 2020 147,950 individuals would be diagnosed with CRC in the United States (70.7% would suffer from colon and 29.3% from rectal cancer) while 53,200 patients would die from the disease. 2021's projection has not improved since it is estimated that 149,500 individuals will be affected and 52,980 will die by it. Although the risk of developing colorectal cancer increases with age—more than 90% of cases occur in people aged 50 or older—recent research shows that the incidence of colorectal

cancer has been increasing 1% to 3% annually for people younger than age 50 while decreasing in older individuals. Since 1994, cases of young-onset colorectal cancer—defined as colorectal cancer diagnosed before the age of 50—have increased by 51%, according to the National Cancer Institute. The alarming rise in the incidence of colorectal cancer in young adults prompted the American Cancer Society (ACS), in 2018, to change its recommendations for the start of colorectal screening from age 50 to age 45 for individuals at average risk. In addition, research by the ACS has found that people younger than age 55 are 58% more likely to be diagnosed with late-stage disease than older adults, making cure more difficult. Although the uptick in colorectal cancer incidence in younger adults correlates with the increasing rates of obesity in the United States, it is unclear what role obesity and other common risk factors, including diet, inactivity, and family history, may be playing in the early onset of this disease. CRC patients with distant metastases present the worst prognosis since a significant number of them develops resistance to their therapy. Unfortunately, diagnosis of chemoresistance is most often delayed, allowing for cancer progression to take place before these patients receive second or third line treatments. Therefore, patients who have developed chemoresistance will be recognized late while they will have suffered the side-effects and costs of an ineffective treatment. At the same time, healthcare systems are dealing with an immense financial burden as a result of these treatments. Therefore, it is crucial to identify accurate, cost efficient and easy-to-use tools that will provide valuable prognostic and predictive information. A great body of evidence indicates molecular biomarkers as promising candidates for this purpose. Therefore, effective and easy-to-use prognostic biomarkers are need. This doctoral thesis focused on validating the clinical and possible prognostic value of two novel biomarkers (MNf, and TA) for laCRC and mCRC. These biomarkers were chosen on the basis of their close relation to chromosomal instability (CIN) and aberrant genetic function; both major hallmarks in colorectal carcinogenesis. A secondary objective was the correlation of these biomarkers with molecular biomarkers already used in clinical practice such as CEA, CA-19.9 and alpha fetoprotein (α-FP). At the first stage, blood collection from healthy individuals who just had a normal colonoscopy was performed. At the same time, CRC patients who were to be treated in at the Department of Medical Oncology, University Hospital of Heraklion were evaluated for participation in this study. Unfortunately, not all of the patients who were evaluated for their participation, were found to be eligible for this study. Moreover, given that this study was performed in single center located in an area with approximately 200.000 residents, the study population is relatively small. At the second stage, standardization of the techniques was performed using peripheral blood from two perfectly healthy individuals, allowing for the full set of both experiments to take place by evaluation. At the third and final stage, medical records of all participants were evaluated and these data were compared against each patients' MNf and TA data. After this stage, statistical analysis was performed (Figure 13).

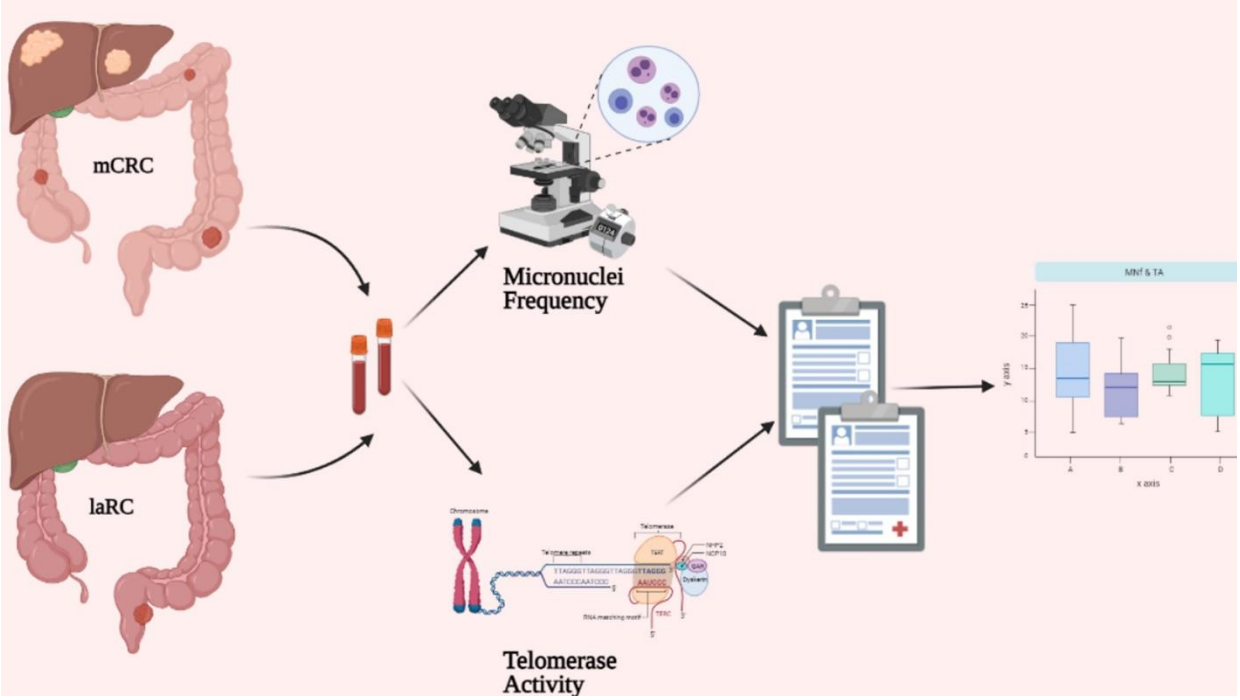

Figure 13. Graphical abstract of this doctoral study. Created with BioRender.com

4. Patients and study protocol

For the needs of this doctoral thesis, 94 consecutive patients treated at the Department of Medical Oncology, University Hospital of Heraklion were evaluated for participation between December 2016 and February 2021.

3.1 Inclusion criteria

In order to create a comparable group of patients certain inclusion criteria were applied. This allowed for consistency among patients' comparisons and scientific solidity as the produced data would be comparable to those generated from other studies.

1) Patients with radiologic evidence of mCRC documented by computed tomography (CT) and/or magnetic resonance imaging (MRI) presenting measurable disease treated with 1st line systemic treatment according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (190)
2) Patients with radiologic evidence of laRC documented by abdominal and chest computed tomography (CT) and abdominal and pelvic magnetic resonance imaging (MRI) presenting measurable disease receiving induction chemotherapy according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (74).

3.2 Exclusion criteria

In order to create a comparable group of patients certain inclusion criteria were applied. This allowed for consistency among patients' comparisons and scientific solidity as the produced data would be comparable to those generated from other studies.

1) Failure to complete the therapeutic regimen for any reason (toxicity, refusal of the patient, or death)
2) Refuse of the patient to attend the study
3) Synchronous second primary cancer at the time of enrollment and/or therapy

Out of 94 patients evaluated, 85 were found eligible and were included however only 76 managed to complete it (55 mCRC and 21 laRC) since 5 patients presented increased toxicity and had to stop while another 4 died prior to completion of the study (Figure 14).

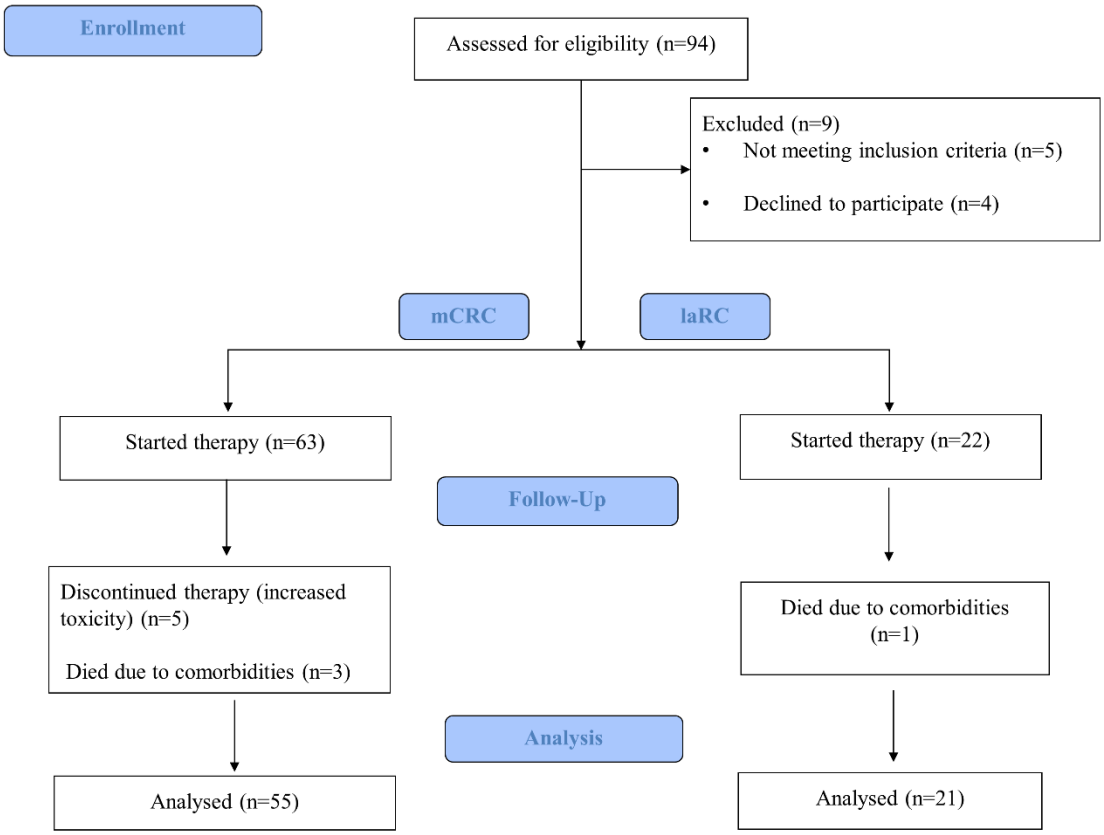

Figure 14. CONSORT flow diagram of this doctoral study

In order to be anatomically and embryologic accurate, the embryologic origin of each part of the colon in order to divide primary location of the lesion in right and left sided (caecum, ascending colon and proximal 2/3 of the transverse colon from the midgut and therefore right colon, distal third of the transverse, the descending, sigmoid colon and the rectum from the hindgut and therefore left colon). All participants signed an informed consent. All samples generated by this study were anonymized and personal data were managed according to the EU General Data Protection Regulation (GDPR).

3.3 Therapy selection for mCRC

Based on the chemotherapeutic protocol that was selected mCRC patients received one of the following therapies: I) folinic acid with 5-fluorouracil and oxaliplatin (FOLFOX) II) folinic acid with 5-fluorouracil and irinotecan (FOLFIRI) or III) folinic acid with 5-fluorouracil, oxaliplatin and irinotecan (FOLFOXIRI). Based on the genetic profile of each patient a biological factor could be used [anti-VEGF (Bevacizumab, Aflibercept) and anti-EGFR (Cetuximab, Panitumumab)].

3.4 Therapy selection for laRC

Patients with laRC were informed about their therapeutic strategy: Induction chemotherapy and then operation for RC. Chemotherapeutic protocols were FOLFOX and capecitabine and oxaliplatin (CAPOX).

3.5 Response evaluation

The RECIST criteria version 1.1 were used as the gold standard for the evaluation of the treatment response (191). At baseline, tumor lesions/lymph nodes were categorized measurable or non-measurable. For tumor lesions in order to be considered as candidates for response follow up they should be accurately measured in at least one dimension (longest diameter in the plane of measurement is to be recorded) with a minimum size of: 10 mm by CT scan (CT scan slice thickness no greater than 5 mm). 10 mm caliper measurement by clinical exam (lesion which cannot be accurately measured with calipers should be recorded as non-measurable). 20 mm by chest X-ray. In case that the lesion of interest is a malignant lymph node then in order to be considered pathologically enlarged and measurable, a lymph node must be 15 mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the short axis will be measured and followed.

All other lesions, including small lesions (longest diameter <10 mm or pathological lymph nodes with P10 to <15 mm short axis) as well as truly non-measurable lesions. Lesions considered truly non-measurable include: leptomeningeal disease, ascites, pleural or pericardial effusion, and inflammatory breast disease, lymphangitic involvement of skin or lung, abdominal masses /abdominal organomegaly identified by physical exam that is not measurable by reproducible imaging techniques.

All measurements should be recorded in metric notation, using calipers if clinically assessed. All baseline evaluations should be performed as close as possible to the treatment start and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging based evaluation should always be done rather than clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

CT is the best currently available and reproducible method to measure lesions selected for response assessment. This guideline has defined measurability of lesions on CT scan based on the assumption that CT slice thickness is 5 mm or less. As is described in Appendix II, when CT scans have slice thickness greater than 5 mm, the minimum size for a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (e.g. for liver lesions).

Using these criteria, patients were evaluated at the end of the therapy and were divided according to their response into four subgroups:

1) Complete response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm.
2) Partial response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.
3) Stable disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study
4) Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (the appearance of one or more new lesions is also considered progression)

3.6 Blood sampling

Using sterile syringe and aseptic techniques, peripheral blood samples were collected at predetermined time-points. For mCRC patients these were before the beginning of the treatment, in the middle of it and at the end of treatment (approximately 0, 3 and 6 months of treatment, respectively). For laRC patients samples were taken before the beginning of chemotherapy and at the end. We also took blood samples two weeks postoperatively from twelve laRC patients. All blood samples were stored in 4 ◦C until processing within 48 hours from sampling.

3.7 Control group

The control group was constituted by 25 healthy individuals with no medical condition after having normal colonoscopy. All individuals provided an informed written consent. Inclusion criteria were: age between 45 and 75 years old, medical history free of cancer, autoimmune diseases, diabetes mellitus type I or II, and chronic obstructive pulmonary disease (COPD), non-smokers or no smoking habits for the last ten years and no consumption of immune-modifying medication. Exclusion criteria were the presence of the above-mentioned diseases, direct exposure at any time (domestic or occupational) to pesticides, herbicides, organic solvents or any persistent organic pollutant, for women the use of oral contraceptives, and the will not to participate in the study.

4. Materials and Methods

For the needs of this doctoral thesis the following experimental procedures were used:

1. Cytokinesis-Block Micronucleus assay in peripheral blood lymphocytes for the evaluation of the genotoxic effect of CRC and chemo/radio therapy upon lymphocytes
2. Telomerase activity using photometric enzyme immunoassay for the detection of telomerase activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP) ELISA

These techniques are thoroughly present below:

4.1 CBMN in peripheral blood lymphocytes (PBLs)

All the necessary equipment used for this process was sterilized using dry heat sterilization process at 200°C for 30 minutes. Cell culture flasks, pipettes and Eppendorf conical tubes were retrieved sterile by the production companies.

The water that was used for the production of Giemsa staining was double distilled and sterile in glass cone vials in autoclave using steam heated to 130 °C under pressure 2 atm for 15 minutes.

All experimental procedures took place in a laminar flow cabinet where UV radiation for 25 minutes was used to disinfect all reagents and equipment used prior every culture setting. Blood sampling was performed using sterile single use syringes after alcohol solution 90% was applied on patients' skin.

Blood samples were transferred in heparin tubes in order to avoid blood clotting and the blood was kept in refrigerator at 4°C for up to 48 hours (until when cell culture should be made).

Blood cultures were incubated in an incubator under stable conditions of 5% CO2 concentration, 30% humidity and 37°C for 72 hours.

4.1.1 Materials

1) Whole blood (0.5 ml)
2) 6.5 ml Ham's F-10 medium (Gibco/Thermo Fisher Scientific, Waltham, MA USA)
3) 1.5 ml fetal bovine serum (Standard Fetal Bovine Serum, certified, US origin, Gibco/Thermo Fisher Scientific)
4) 0.3 ml phytohemagglutinin M (PHA-M; 10 ml, Thermo Fisher Scientific)
5) 6μg/ml or 25 μl of cytochalasin-B (white to off-white powder, ≥98% 5 mg; Acros Organics, Inc./Thermo Fisher Scientific) was added 44 h following culture initiation
6) 0.3 ml Penicillin-Streptomycin (10,000 U/mL) (Gibco/Thermo Fisher Scientific, Waltham, MA USA)

4.1.2 Solutions

1) Hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v)
2) Fixative solution of methanol:acetic acid (5:1, v/v)
3) Sorensen's phosphate buffer: 9.08 gr of Monopotassium phosphate (KH_2PO_4) (83.7mM) and 11.88gr of sodium phosphate dibasic (Na_2HPO_4) in 1 liter of double distilled water (66.7mM). The resulting solution was Na_2HPO_4 / KH_2PO_4 1:1 with pH 7.2
4) Giemsa (Gibco/Thermo Fisher Scientific) 15% (dilution in Sorensen's phosphate buffer)
5) Cytochalasin b 5 mg in 500 μl of Dimethyl sulfoxide (DMSO) [$(CH_3)_2SO$]

4.1.3 Methodology

The methodology of peripheral blood lymphocytes (PBLs) cultivation for the identification of micronuclei was based upon the proposed methods of CBMN with

use of cytochalasin b while standard criteria for micronuclei evaluation were used as these were proposed by Fenech (2003). In detail, the cultivation protocol stands as follows:

1) In a laminar flow cabinet where UV radiation for 25 minutes was used to disinfect all reagents and equipment prior culture. In each flask, 0.5 ml of whole blood to 6.5 ml Ham's F-10 medium, 1.5 ml fetal bovine serum, 0.3 ml phytohemaglutinin M and 0.3 ml of Penicillin-Streptomycin were added. Afterwards, blood cultures were incubated at 37°C, with 5% CO_2 and 30% humidity for a period of 72 h.
2) At 44 hours after culture initiation, flasks were taken under aseptic conditions from the incubator to the laminar flow cabinet where six micrograms per milliliter corresponding to 25 microliters of cytochalasin-B were added. After that, each flask was brought back to the incubator for another 28 hours.

At 72 hours post-incubation, culture of PBLs has finished and the following steps are applied in order to achieve PBL isolation according to the technique of Rothfels and Siminovitch 1958:

1) After 72 in the incubator, the blood solution contained in each flask is transferred to separate 15 ml sterile conical polypropylene centrifuge tubes. Then, centrifuge tubes are centrifuged at 400 × g (1,500 rpm) at 20°C for 10 minutes.
2) After the completion of centrifugation, supernatant (approximately 7 ml) is removed using a sterile plastic pipette and 4 ml of the hypotonic solution (Ham's F-10 medium and milli-Q water 1:1, v/v) are added. The conical centrifuge tube is stirred using a vortex laboratory shaker at 2000 rpm for 10 seconds and then is left aside for 2 minutes.
3) 6 ml of the fixative solution of methanol:acetic acid (5:1, v/v) are added slowly while stirring the tube on the vortex laboratory shaker at 2000 rpm
4) Conical centrifuge tubes are put back to the centrifuge at 400 × g (1,500 rpm) at 20°C for 10 minutes.
5) We remove the supernatant (approximately 7 ml) and 6 ml of the fixative solution of methanol:acetic acid (5:1, v/v) are added slowly while stirring the tube on the vortex laboratory shaker at 2000 rpm
6) Conical centrifuge tubes are put back to the centrifuge at 400 × g (1,500 rpm) at 20°C for 10 minutes
7) Steps 5,6 are repeated until the solution is clear (approximately 2-3 times)
8) Since the solution has become clear, we remove the supernatant so that less than 0.5 ml is left. We mobilize the cellular pellet with a pipette and drop 2-3 drops of the cellular solution over clean microscope slides. Slides are left to air-dry at room temperature
9) Having the slides dried, we immerse them in Giemsa 15% solution with Sorensen's phosphate buffer at 25°C for 20 min
10) We leave microscope slides to air-dry at room temperature and afterwards we apply two drops of mounting medium and place a cover slip over each slide. The slides were then placed under a Nikon Eclipse E200 microscope (Nikon Holdings Europe B.V., Amsterdam, The Netherlands) where the binucleated cells (BN cells) and MN were viewed. One thousand BN cells with an intact

cytoplasm were scored per slide for each sample, in order to calculate the MNf (Figure 15).

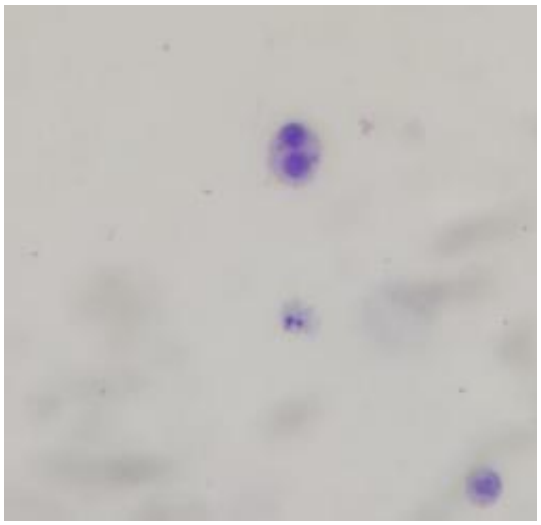

Figure 15. Indicative MN in binucleated cell photographed from a cell culture

4.2 Telomerase activity estimation using photometric enzyme immunoassay utilizing the Telomeric Repeat Amplification Protocol (TRAP) polymerase chain reaction (PCR) enzyme linked immune sorbent assay (ELISA)

All the necessary equipment used for this process was sterilized using dry heat sterilization process at 200°C for 30 minutes. Pipettes and Eppendorf conical tubes were retrieved sterile by the production companies.

All experimental procedures took place in a laminar flow cabinet where UV radiation for 25 minutes was used to disinfect all reagents and equipment used prior blood handling. Blood sampling was performed using sterile single use syringes after alcohol solution 90% was applied on patients' skin.

Blood samples were transferred in K2EDTA tubes in order to avoid blood clotting and the blood was kept in refrigerator at 4°C for up to 48 hours (until when separation of peripheral blood lymphocytes should be made).

PBS is an isotonic buffer frequently used in biological applications, such as washing cells, transportation of tissues, and dilutions. PBS closely mimics the pH, osmolarity, and ion concentrations of the human body. Since it is nontoxic to cells, it is extensively used for cell container rinsing and other preparations that might leave a residue. It is simple to prepare and has good shelf life, but will precipitate in the presence of zinc ions.

Eppendorf conical tubes containing peripheral blood lymphocytes were kept in deep freezer (-80°C) until TRAP-ELISA assay could be done.

The determination of telomerase activity in the PBMCs was performed quantitatively using the teloTAGGG telomerase PCR ELISA PLUS kit (Roche Diagnostic GmbH, Mannheim, Germany). A total of 2×105 cells were transferred to a PCR tube for sample preparation for telomerase activity assay. Following centrifugation at 3,000 × g for 10 min, the supernatant was carefully removed and the pellets were collected. The assay principle was consisting of two steps. The first step included the addition of

telomeric repeats (TTAGGG) to the 3′ end of biotin-labelled primers and product amplification by PCR. The second step consisted of ELISA reaction. The workplaces for sample preparation, TRAP reaction, and detection of amplicons were physically separated in order to minimize the risk of carry-over contamination. In order to achieve higher data reliability all samples were tested in triplicates. TA was expressed as a total and as per outcome (progression, stable, partial and complete response).

4.2.1 Materials for peripheral blood mononuclear cells (PBMCs) harvesting

1) 2.5 ml of whole blood
2) 32.5 ml Phosphate-buffered saline (PBS) 1x
3) 5 ml Ficoll- Histopaque®-1077 (Gibco/Thermo Fisher Scientific, Waltham, MA USA)

4.2.2 Solutions

1) PBS 1x, (1000 ml of Milli-Q® Water, 8 g of NaCl, 200 mg of KCl, 1.44 g, Na_2HPO_4, 245 mg of KH_2PO_4). pH is adjusted to ≈ 7.4

4.2.3 Methodology

The methodology of peripheral blood mononuclear cells (PBMCs), that is monocytes and lymphocytes, isolation follows these steps:

1) 2.5 ml of whole blood are transferred with a pipette in a 15 ml sterile conical polypropylene centrifuge tube and 2.5 ml of PBS 1x are added. The solution of blood/PBS (1:1, v:v) is gently mixed
2) 5 ml Ficoll- Histopaque®-1077 are transferred with a pipette in a 15 ml sterile conical polypropylene centrifuge tube and the solution of blood/PBS is carefully added with a pipette in an incline so that the solution is not mixed the Ficoll- Histopaque®-1077 (Figure 16)
3) Conical centrifuge tube is then put to the centrifuge at 1600 rpm at 20°C for 25 minutes
4) The cloudy band between supernatant (plasma) and Ficoll- Histopaque is carefully transferred with a pipette to a new 15 ml sterile conical polypropylene centrifuge tube avoiding any Ficoll- Histopaque contamination (Figure 16)
5) The cellular solution is mixed with 10 ml of PBS 1x and then the tube is centrifuged at 2000 rpm at 20°C for 5 minutes
6) The supernatant is removed and 10 ml of PBS 1x is added. Cellular pellet is mobilized with pipette suctioning. The tube is centrifuged at 2000 rpm at 20°C for 5 minutes
7) The supernatant is removed and the pellet is transferred with a pipette in an Eppendorf conical tube where 1.5 of PBS 1x is added. The tube is centrifuged at 2000 rpm at 20°C for 5 minutes
8) The supernatant is removed and the Eppendorf conical tube is put in deep freezing (-80°C) until TRAP-ELISA assay is performed

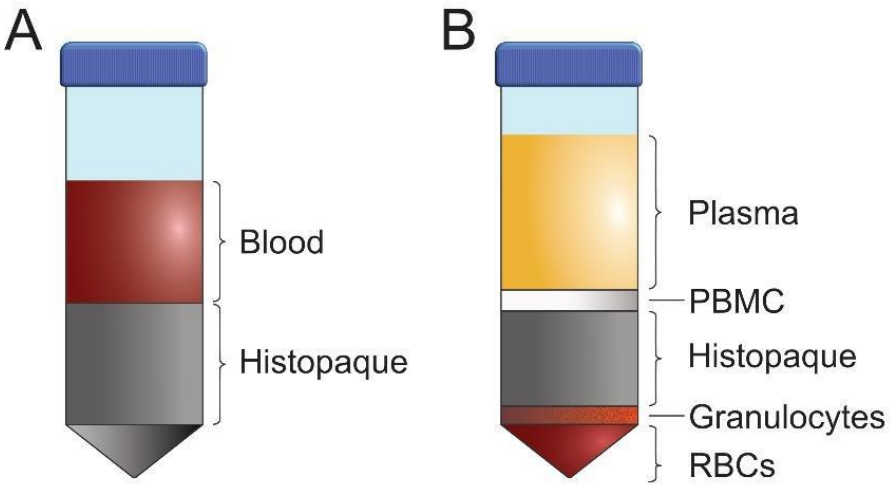

Figure 16. Graphical presentation of the key steps in lymphocytes isolation

4.2.4 Materials for Telomeric Repeat Amplification Protocol (TRAP) polymerase chain reaction (PCR) enzyme linked immune sorbent assay (ELISA)

1) 500 ml of Milli-Q® Water
2) Microcentrifuge
3) PCR thermal cycler
4) Tubes for PCR amplification
5) Sterile reaction (Eppendorf) cups for preparing dilutions
6) Heatable Microplate shaker
7) Microplate reader
8) Buffers in a Box, Premixed PBS, 10× RNase, DNase-free

4.2.5 Solutions

1) Washing buffer, 1×: Dilute an appropriate volume of Washing buffer, 10× conc. (bottle 5) with autoclaved double dist. water (1:10) and mix thoroughly. Approx. 2.5 ml of the diluted Washing buffer are needed for one reaction
2) Anti-DIG POD, stock solution: Reconstitute the lyophilizate in 240 µl autoclaved double dist. water. This results in an antibody conjugate concentration of 0.5 U/ml
3) Anti-DIG POD, working solution: To prepare the working solution, dilute an appropriate amount of the reconstituted anti-DIG-POD (solution 6) with Conjugate dilution buffer (solution 7) to a final concentration of 10 mU/ml (e.g., 100 ml antibody solution and 4.9 ml of Conjugate dilution buffer).
4) Positive control, cell extract: Reconstitute lyophilized cell extract on ice with 20 ml autoclaved double dist. water and mix thoroughly. The reconstituted solution has a concentration of about 1×10^3 cell equivalents per microliter. Dispense solution into suitable aliquots (1–3 ml will be needed for 1 reaction). Keep the extract on ice during pipetting.
5) Preparation of negative control: Telomerase essentially requires integrity of its internal RNA component as a template for the addition of the telomeric repeat sequences to the telomerase-specific primer. Therefore, pre-incubation of the cell or tissue extract with RNase, DNase-free will fully destroy telomerase activity contained in the extract and offers a convenient control of specificity.

4.2.6 Methodology

The following protocol was used to provide a positive control:

1) 5 µl of cell extract typically corresponding to 5000 cell equivalents are incubated with 1 µl DNase-free RNase (1 µg/µl) for 20 min at 37°C.
2) An aliquot of 1–3 µl of the RNase treated extract is used for the TeloTAGGG Telomerase PCR ELISA.

Note: Incubation of a telomerase-positive extract at 37°C for 20 min in the absence of RNase does not affect the telomerase-specific signals. Hence any loss of signal is due to specific degradation of the internal RNA component. Exert extreme care to avoid carry-over of RNase into samples, resulting in false-negative readings. Alternatively, heat-treatment of the cell extract for 10 min at 85°C prior to the TRAP reaction may be used to inactivate telomerase protein for producing negative controls.

Preparation of extracts from cells

1) Harvest and count cells using a hemocytometer, Neubauer
2) Transfer 2 × 105 cells per single reaction into a fresh Eppendorf tube.
3) Pellet cells at 3000 × g for 10 min in a refrigerated centrifuge at +2 to +8°C
4) Carefully remove supernatant and resuspend the cells in PBS and repeat the centrifugation step
5) Carefully remove the supernatant. If the TeloTAGGG Telomerase PCR ELISA is not performed immediately after the extract preparation, the pelleted cells can be stored at -80°C until use
6) Resuspend the pelleted cells in 200 µl Lysis reagent (solution 1), pre-cooled on ice by retropipetting at least 3 times and incubate on ice for 30 min.

Note: Because frozen cell pellets were used for extraction, thaw cell pellets on ice before adding Lysis reagent (solution 1)

7) Centrifuge the lysate at 16.000 × g for 20 min at +2 to +8°C (in a refrigerated centrifuge)
8) Carefully remove the supernatant and transfer to a fresh tube. To ensure that no cellular debris of the pelleted cells is transferred, we recommend pipetting only 175 µl of the cell extract

Telomeric repeat amplification protocol (TRAP reaction)

4) For each sample to be tested and the controls, transfer 25 µl Reaction mixture (Solution 2) into a tube suitable for PCR amplification

5) Samples: 1–3 µl cell extract per tube (corresponding to $1\times 10^3 – 3\times 10^3$ cell equivalents or 1– 50 µg total protein, see sample preparation)

6) Negative controls: 1–3 µl of the corresponding RNase treated or heat treated cell extract (corresponding to $1\times 10^3 – 3\times 10^3$ cell equivalents or 1 –50 µg total protein)

7) Positive control: 1–3 µl of the reconstituted solution (corresponding to 1×10^3 cell equivalents)

Note: All pipetting steps should be done on ice

8) Transfer tubes to a thermal cycler and perform a combined primer elongation/amplification reaction by the protocol in Table 4:

Phase	Time	Temp.	Cycles
Primer elongation	10-30 min	25°C	1
Telomerase inactivation	5 min	94°C	1
Amplification: Denaturation Annealing Polymerization	30s 30s 90s	94°C 50°C 72°C	1-30
	10 min	72°C	1
Hold		4°C	

Table 4. Elongation/amplification reaction by the protocol

Hybridization and ELISA procedure

1) Per sample transfer 20 µl of Denaturation reagent (solution 3) into a nuclease-free, uncoated microplate (MP)
2) Add 5 µl of the amplification product and incubate at +15 to +25°C for 10 minutes
3) Add 225 µl Hybridization buffer (solution 4) per tube and mix thoroughly by vortexing briefly.
4) Transfer 100 µl of the mixture per well of the precoated MP modules supplied with the kit and cover the wells with the self-adhesive cover foil to prevent evaporation
5) Incubate the MP modules at 37°C on a shaker (300 rpm) for 2 hours
6) Remove the Hybridization solution completely. Wash 3 times with 250 µl of Washing buffer (solution 5) per well for a minimum of 30 s each and remove Washing buffer carefully
7) Add 100 µl Anti-DIG-POD working solution (solution 11) per well. Cover the MP modules with a cover foil and incubate at 15 –25°C (18–22°C) for 30 minutes while shaking at 300 rpm
8) Remove the solution completely. Rinse 5 times with 250 µl of Washing buffer (solution 5) per well for a minimum of 30 seconds each, and remove Washing buffer carefully
9) Add 100 µl TMB substrate solution (solution 8) prewarmed to room temperature per well. Cover the wells with cover foil and incubate for color development at +15 to +25°C for 10–20 minutes while shaking at 300 rpm
10) Without removing the reacted substrate, add 100 µl Stop reagent (solution 9) per well to stop color development. Addition of the Stop reagent causes the reacted POD substrate to change in color from blue to yellow, and is required to achieve maximal sensitivity (Figure 17).

11) Using a Microplate (ELISA) reader, measure the absorbance of the samples at 450 nm (with a reference wavelength of approx. 690 nm) within 30 min after addition of the Stop reagent
12) Absorbance values are reported as the A450 nm reading against blank (reference wavelength A690 nm).

Negative control An appropriate negative control for checking the specificity of the telomerase reaction is degrading the telomerase-associated RNA by preincubating the cell extract with DNase-free RNase. Alternatively, heat-treatment may be used to prepare negative controls. With this RNase treatment, the maximium value of absorbance for the negative control should be 0.25 A450 nm – A690 nm units. If values are higher, the whole experiment including TRAP reaction should be repeated.

Positive control The absorbance readings obtained with the positive control (supplied with the kit) should be higher than 1.5 A450 nm – A690 nm units after 20 min substrate reaction, when using 1 × 103 cell equivalents in the assay. If values are lower, the whole experiment including TRAP reaction should be repeated.

Samples Subtract the mean of the absorbance readings of the negative controls from those of the samples. Samples are regarded as telomerase-positive if the difference in absorbance (ΔA) is higher than 0.2 A450 nm – A690 nm units.

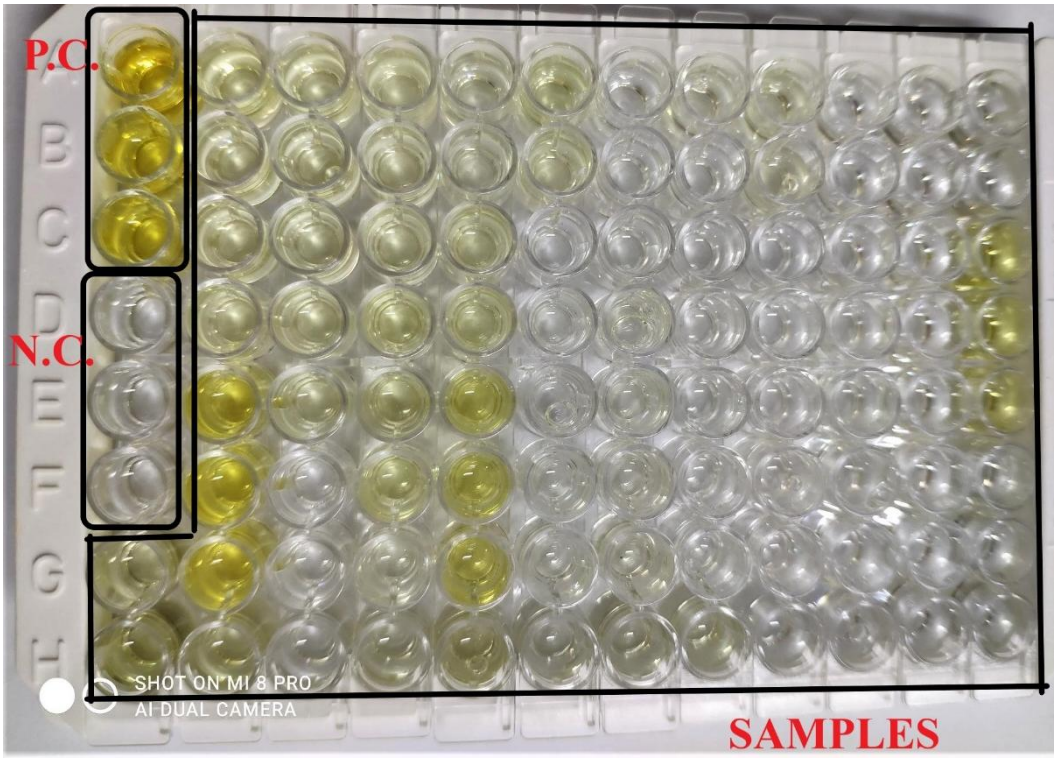

Figure 17. Indicative photograph from a TRAP-ELISA experiment of this doctoral study. P.C. = Positive control, N.C.= negative control

4.3 Statistical analysis

Frequency data were analysed using non-parametric statistics. Pearson's Chi-square test (χ^2) was applied to estimate differences in proportions of patients' and disease characteristics (Table 1). In order to examine TA differences and percentage differences of MNf (%DMNf) between two groups (e.g mCRC vs IaRC), the Mann-Witney test was applied. Whereas, in order to examine TA differences and %DMNf

for more than two groups (e.g. disease response) Kruskal-Wallis comparisons were applied. Comparison of counts of MNf were assessed using G-test when bivariate comparisons of before, middle and after therapy sampling points were compared. The Chi-squared test was used for the analysis of the CBPI values. A crude, due to small number of cases, discrimination limit between responses was established using %DMNf as an indicator. The %DMNf definition between middle and before was set by the formula

$$\%DMNf = \frac{MNf_{middle} - MNf_{before}}{MNf_{before}} 100\%$$

ROC curve analysis, corresponding diagrams of sensitivity vs 1-specificity were applied between %DMNf in a binary response (progressive vs stable/partial/complete response and progressive/stable vs partial/complete response) according to %DMNf.

TA was measured in 28 patients, whose characteristics are presented in Table 1, at three time points: before (at the beginning of the therapy), middle (at the middle of therapy for mCRC and at the end of therapy for laRC) and after (at the end of therapy for mCRC and after surgery for laRC).

IBM SPSS Statistics 26.0 and OpenEpi 3.01 open source epidemiological program (https://www.openepi.com/Menu/OE_Menu.htm) were used for statistical analysis of data and sensitivity analysis. A level of 0.05 was set as level of acceptance.

5. Results

As presented in Table 5, for the needs of this doctoral thesis 76 CRC patients were prospectively studied. Out of these 76 patients, 55 were diagnosed with mCRC and 21 with laRC. In total, 44 female patients (57.9%) and 32 male patients (42.1%) were included. For the mCRC group, sex groups were almost equally proportionate (females 27 and males 28, 49.1% and 50.9% accordingly). In the laRC group there was a predominance of the male patients since there were 16 males against 5 females (76.2% and 23.8% accordingly). However, in mCRC group there were more females than those in laRC (27 and 5 accordingly, p=0.034) highlighting that laRC is not as common as mCRC. Regarding the age distribution, most patients were aged between 56-70 years old (32 cases, 42.1%) and this was also observed for the mCRC (24, 43.6%) and laRC groups (8, 38.1%) (p=0.435) (Table 6). Tables 7-10 provide a thorough presentation of each patients' characteristics [age, sex, therapy, biologic agent (if given), disease response and mutation status (when this was known)]

5.1 metastatic CRC (mCRC) In the mCRC group, 29 patients were treated with FOLFOX, 22 with FOLFIRI and four with FOLFOXIRI (52.7%, 40% and 7.3% respectively). Moreover, 40 patients received an additional treatment with a biological agent (cetuximab, aflibercept, bevacizumab or panitumumab) based on their genetic profile; 19 were treated with Bevacizumab, five with Aflibercept, nine with Cetuximab and seven with Panitumumab (34.5%, 9%, 16.3% and 12.7% accordingly). Based on RECIST criteria for disease response evaluation, the generated data suggest that the mCRC group had the following characteristics: 13 patients exhibited progressive disease, 18 stable disease, 23 had partial response while one had complete

response (23.6%, 32.7%, 41.8% and 1.8% respectively). Finally, 32 of the mCRC patients had left-sided primary lesion (76.4%) while 13 had right sided (23.63%).

5.2 Locally advanced Rectal Cancer (laRC) In the laRC group 12 were treated with FOLFOX (57%) and 9 with CAPOX (43%). Based on RECIST criteria for disease response evaluation, the generated data suggest that the laRC group, two patients had progressive disease, five stable, 11 presented partial response and three had complete response (9.5%, 23.8%, 52.4% and 14.3% respectively).

		MNf			TA		
		mCRC	**laRC**	**p**	**mCRC**	**laRC**	**P**
Total Number of patient		55	21		23	5	
Sex, n (%)	**Males**	28 (50.9)	16 (76.2)	0.068	9 (39.0)	3 (60.0)	0.393
	Females	27 (49.1)	5 (23.8)		14 (61.0)	2 (40.0)	
Age, n (%)	**≤40**	0 (0.0)	1 (4.8)	0.435	1 (4.8)	1 (20.0)	0.173
	41-55	13 (23.6)	5 (23.8)		5 (23.8)	3 (60.0)	
	56-70	24 (43.6)	8 (38.1)		8 (38.1)	1 (20.0)	
	70+	18 (32.7)	7 (33.3)		7 (33.3)	0 (0.0)	
ECOG performance status, n (%)	**0**	22 (95.6)	47 (85.5)	0.199	19 (86.4)	5 (100.0)	0.08
	1	1 (4.3)	8 (14.5)		3 (13.6)	0 (0.0)	
Chemotherapy, n(%)	**FOLFOX**	29 (52.7)	12 (57.0)	0.98*	10 (43.5)	4 (80.0)	0.14*
	FOLFIRI	22 (40.0)	0 (0.0)		13 (36.3)	0 (0.0)	
	FOLFOXIRI	4 (7.3)	0 (0.0)		0 (0.0)	0 (0.0)	
	CAPOX	0 (0.0)	9 (43.0)		0 (0.0)	1 (20.0)	
Biological Agent, n (%)	**Bevacizumab**	19 (34.5)		NA	7 (30.0)		NA
	Aflibercept	5 (9.0)			5 (22.0)		
	Cetuximab	9 (16.5)			6 (26.0)		
	Panitumumab	7 (12.7)			0 (0.0)		
	No agent	15 (27.2)			5 (22.0)		
Disease Response, n (%)	**Progression**	13 (23.6)	2 (9.8)	0.08	6 (20.0)	0 (0.0)	0.25
	Stable disease	18 (32.7)	5 (23.8)		7 (30.0)	3 (60.0)	
	Partial response	23 (41.8)	11 (52.4)		9 (40.0)	1 (20.0)	
	Complete response	1 (1.8)	3 (14.3)		1 (4.0)	1 (20.0)	
KRAS, n (%)	**WT**	25 (45.5)	9 (39.1)	0.61**	Unknown		
	exon 2 mut	18 (32.7)	6 (26.1)		***		
	exon 3 mut	0 (0.0)	0 (0.0)				
	exon 4 mut	1 (1.8)	1 (4.3)				
	Unknown	11 (20.0)	7 (30.4)				
NRAS, n (%)	**WT**	42 (76.4)	15 (65.2)	0.31**	Unknown		
	Mutation	2 (3.6)	2 (8.7)				
	Unknown	11 (20.0)	6 (26.1)				
BRAF, n (%)	**WT**	41 (74.5)	15 (65.2)	0.48**	Unknown		
	V600E mut	4 (7.3)	1 (4.3)				
	Unknown	11 (20.0)	7 (30.4)				
Mismatch repair	**Proficient**	12 (58.2)	21 (91.3)	0.01	Unknown		

status, n (%)	**Deficient**	2 (3.6)	1 (4.3)				
	Unknown	21 (38.2)	1 (4.2)				
Location of primary lesion, n (%)	**Left**	42 (76.4)	19 (83.0)	0.54	21 (100)	5 (100)	NA
	Right	13 (23.6)	4 (17.0)		0 (0.0)	0 (0.0)	
Metastatic sites [median/mean (range)]	**Liver**	3.6/4.4 (0-20)		NA			NA***
	Lung	3.2/3.5 (0-11)					
	Lymph nodes	0/3.2 (0-14)			0/3.2 (0-6)		
	Peritoneum	0/3.6 (0-8)					

*Table 5. Patient characteristics for MNf and TA groups are presented [sex, age, ECOG performance status, chemotherapy, biologic agent, disease response based on the RECIST criteria, KRAS status, NRAS status, BRAF status, mismatch repair (MMR) status, location of the primary lesion, number of metastatic sites]. * p-values from comparison of FOLFOX with the rest of chemotherapies, ** p-values from comparison between Wild-type and mutations,*** Unknown: There were no data , **** NA: Not applicable*

		Colorectal Cancer						
		laRC		**mCRC**		**Total**		**p**
		n	**%**	**n**	**%**	**N**	**%**	
Sex	**Female**	5	23.8%	27	49.1%	32	42.1%	0.034
	Male	16	76.2%	28	50.9%	44	57.9%	
Age groups	**<= 40**	1	4.8%	0	0.0%	1	1.3%	0.435
	41 - 55	5	23.8%	13	23.6%	18	23.7%	
	56 - 70	8	38.1%	24	43.6%	32	42.1%	
	70+	7	33.3%	18	32.7%	25	32.9%	

Table 6. Patients' distribution according to their age and sex

In Figure 18 the distribution of therapies in colorectal metastatic with or without the presence of biological agent is shown. Patients with mCRC were primarily treated with FOLFOX (29 patients, 52.7%), and FOLFIRI (22 patients, 40.0%), while only 4 of them (7.3%) were treated with FOLFOXIRI. On the other hand, laRC patients were primarily treated with FOLFOX (12 cases, 57.1%). Two of 4 patients (50.0%) treated with FOLFOXIRI, 21 of 29 (72.4) treated with FOLFOX and 17 of 24 patients (77.3%) treated with FOLFIRI have an additional biological agent. Further comparative analysis was conducted between mCRC and laRC groups for those characteristics where no data were missing. All comparisons showed that there is an adjustment in demographics and patient's data with the exception of mismatch repair status in MNf dataset (p=0.01). Figure 19 depicts all patients based on their response to the therapy they were given (progressive disease, stable disease, partial response and complete response). As evident by these data, a sum of 38 patients had partial or complete response to their therapy, out of which 24 were mCRC patients. On the

other hand, 2 patients with laRC patients exhibited progressive disease (9.5%), 5 patients exhibited stable disease (23.8%), 11 exhibited partial response (52.4%) while 3 laRC patients had complete response (14.3%). Metastatic colorectal Ca patients showed a reverse profile on outcomes. For example 13 had progressive disease (13.6%), 18 had stable disease (22.7%), 23 presented partial response (41.8%) and only 1 mCRC patient was documented to have complete response (1.9%).

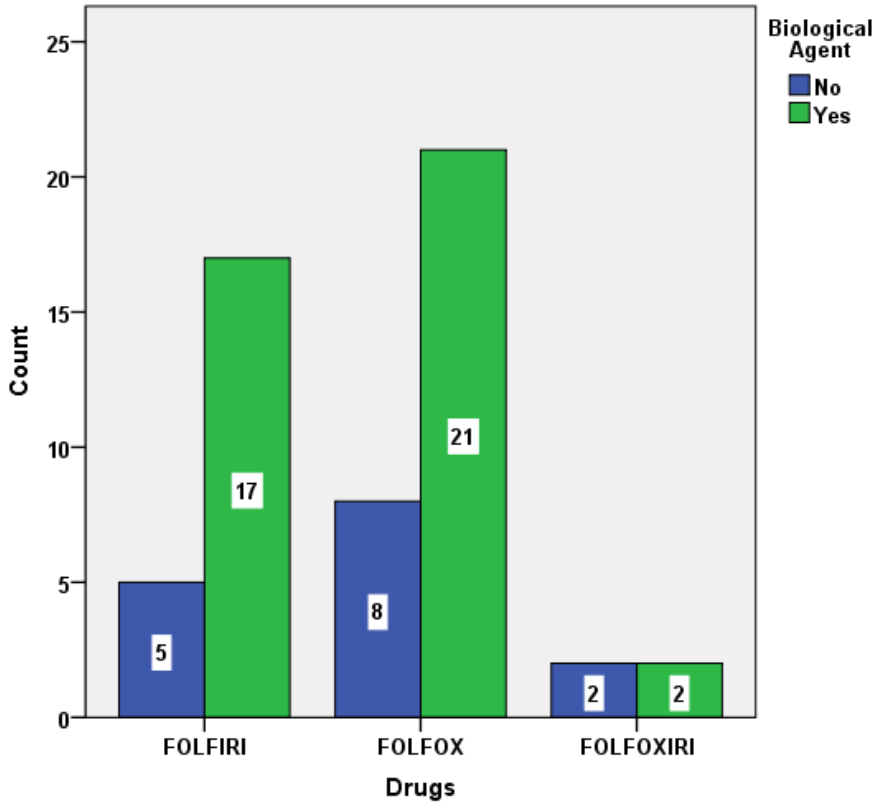

Figure 18. Distribution of used therapies with existence of biological agent in metastatic colorectal patients.

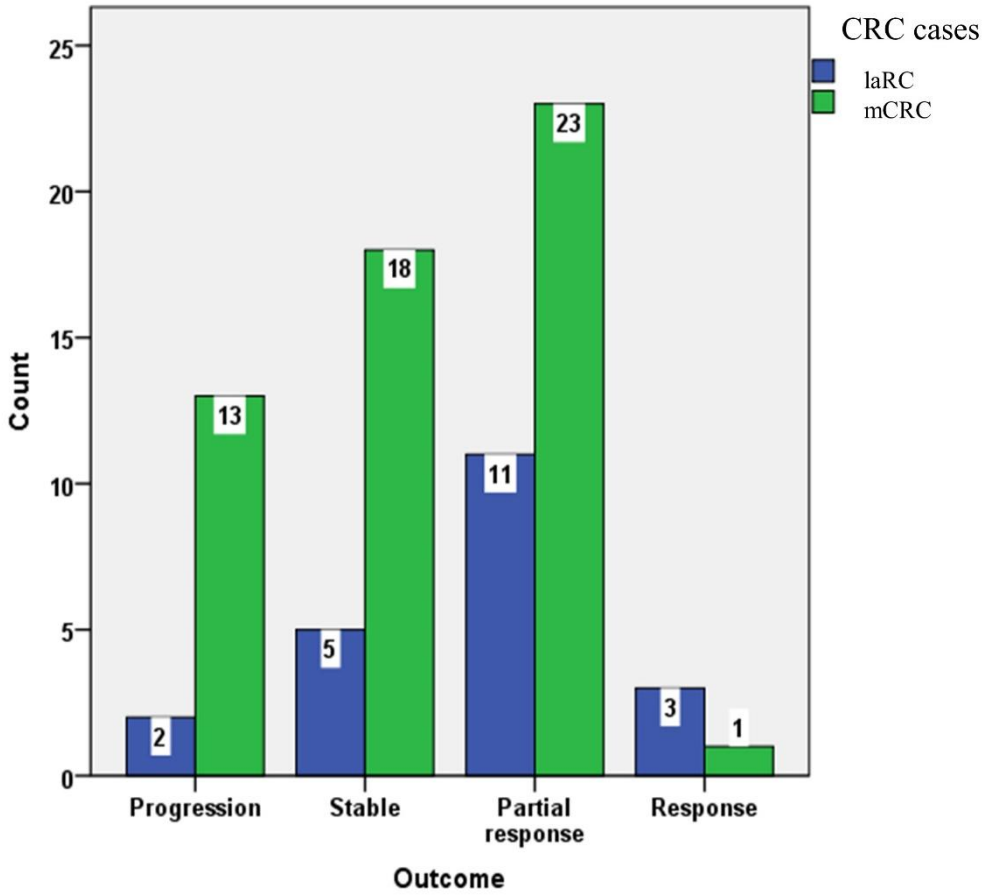

Figure 19. Outcomes of mCRC and laRC patients.

Patient	Age	Sex	Therapy	Response
1	47	female	FOLFOX	Stable disease
2	56	male	CAPOX	Complete response
3	38	female	FOLFOX	Stable disease
4	50	male	FOLFOX	Partial response
5	78	female	FOLFOX	Partial response
6	43	male	CAPOX	Partial response
7	61	male	FOLFOX	Stable disease
8	65	male	FOLFOX	Stable disease
9	45	male	FOLFOX	Stable disease
10	48	female	FOLFOX	Partial response
11	61	male	CAPOX	Partial response
12	60	male	CAPOX	Partial response
13	60	male	CAPOX	Complete response
14	83	male	CAPOX	Progression
15	62	male	CAPOX	Partial response
16	72	male	CAPOX	Progression
17	80	female	FOLFOX	Partial response
18	80	male	FOLFOX	Partial response
19	73	male	FOLFOX	Partial reponse
20	79	male	FOLFOX	Partial
21	66	male	CAPOX	Complete response

Table 7. Detailed presentation of the characteristics of the laRC group

laRC Patients	CEA Before	CEA End	CEA After	Ca19-9 Before	Ca19-9 End	Ca19-9 After
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	2,66	2,3	-	16,5	18	-
4	-	-	-	-	-	-
5	4,2	-	-	-	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	1,68	1,4	-	<0,6	2,6	-
10	-	-	-	-	-	-
11	-	-	-	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	2,64	2	-	-	5	-
15	16	9	2,32	-	<2	-
16	2,14	5,88	-	-	-	-
17	2,07	-	-	-	-	-
18	24	-	-	-	-	-
19	3,95	-	-	10,4	-	-
20	3,7	5,96	-	9,8	10,9	-
21	-	-	-	-	-	-

Table 8. CEA and CA-19.9 levels for laRC patients before the beginning of their therapy, at the end of it and after surgery

Patient	Age	Sex	Therapy	Bio. Agent	Location	Response	Mutation
22	76	male	FOLFOX	Cetuximab	LEFT	Progression	all wt
23	83	female	FOLFIRI	NO	RIGHT	Partial response	BRAF mut
24	80	male	FOLFOX	Cetuximab	LEFT	Progression	all wt
25	44	male	FOLFIRI	Bevacizumab	LEFT	Stable disease	KRAS mut
26	84	female	FOLFIRI	Bevacizumab	RIGHT	Progression	KRAS mut
27	57	female	FOLFIRI	NO	LEFT	Partial response	no data
28	66	male	FOLFOX	Cetuximab	LEFT	Progression	all wt
29	79	female	FOLFIRI	NO	LEFT	Partial response	no data
30	61	female	FOLFIRI	AFLIBERCEPT	LEFT	Stable disease	no data
31	66	female	FOLFIRI	AFLIBERCEPT	LEFT	Partial response	MSI high, BRAF mut
32	70	female	FOLFIRI	AFLIBERCEPT	LEFT	Partial response	no data
33	45	male	FOLFOX	AFLIBERCEPT	LEFT	Progression	NRAS mut
34	47	male	FOLFOX	Cetuximab	LEFT	Stable disease	all wt
35	42	male	FOLFIRI	Cetuximab	RIGHT	Partial response	no data
36	46	female	FOLFOX	Bevacizumab	LEFT	Progression	KRAS mut
37	67	female	FOLFOX	NO	LEFT	Stable disease	no data
38	67	male	FOLFIRI	AFLIBERCEPT	LEFT	Stable disease	NRAS mut
39	65	female	FOLFIRI	Cetuximab	RIGHT	Stable disease	all wt
40	59	female	FOLFIRI	Bevacizumab	LEFT	Partial response	KRAS mut
41	85	female	FOLFOX	NO	RIGHT	Partial response	all wt
42	77	male	FOLFOX	Bevacizumab	LEFT	Stable disease	KRAS mut

43	71	male	FOLFOX	Bevacizumab	LEFT	Partial response	KRAS mut
44	69	male	FOLFOX	NO	RIGHT	Progression	no data
45	69	female	FOLFOX	NO	LEFT	Progression	no data
46	53	female	FOLFOX	Panitumumab	LEFT	Stable disease	all wt
47	74	male	FOLFOX	Panitumumab	LEFT	Partial response	all wt
48	78	male	FOLFOX	NO	LEFT	Partial response	KRAS mut (A146T exon 4)
49	71	male	FOLFIRI	NO	RIGHT	Progression	KRAS mut (G12P)
50	68	female	FOLFOX	NO	LEFT	Partial response	no data
51	43	male	FOLFOX	Bevacizumab	LEFT	Stable disease	KRAS mut (G12P)
52	46	female	FOLFOXIRI	NO	LEFT	Stable disease	no data
53	59	female	FOLFIRI	NO	LEFT	Complete resposne	all wt
54	77	female	FOLFOX	Bevacizumab	LEFT	Partial response	KRAS mut
55	74	female	FOLFOX	Bevacizumab	RIGHT	Stable disease	KRAS mut (G12S)
56	59	male	FOLFIRI	Panitumumab	LEFT	Stable disease	all wt
57	73	male	FOLFIRI	Bevacizumab	LEFT	Partial response	KRAS mut (G13D)
58	76	male	FOLFOX	Panitumumab	LEFT	Partial response	all wt
59	69	male	FOLFIRI	Bevacizumab	LEFT	Partial response	KRAS mut
60	59	male	FOLFOX	Panitumumab	LEFT	Stable disease	all wt
61	55	female	FOLFOXIRI	Bevacizumab	LEFT	Progression	KRAS mut(exon 4 G436A)
62	83	female	FOLFOX	Cetuximab	LEFT	Partial response	all wt
63	67	male	FOLFOX	Cetuximab	LEFT	Stable disease	all wt
64	59	male	FOLFOX	Bevacizumab	RIGHT	Stable disease	KRAS mut (G12D)
65	61	male	FOLFIRI	Bevacizumab	LEFT	Stable disease	KRAS mut (G12D)
66	58	female	FOLFOX	NO	RIGHT	Partial response	BRAFmut (V600E)
67	55	female	FOLFIRI	Panitumumab	LEFT	Partial response	all wt
68	72	male	FOLFIRI	Panitumumab	LEFT	Progression	all wt
69	67	male	FOLFIRI	Bevacizumab	RIGHT	Partial response	KRAS mut (G12D)
70	47	female	FOLFOX	Bevacizumab	LEFT	Progression	KRAS mut (G12S)
71	60	male	FOLFOX	Cetuximab	RIGHT	Partial response	all wt
72	54	female	FOLFOX	Bevacizumab	LEFT	Partial response	NRAS mut (Q61R)
73	61	male	FOLFOX	NO	RIGHT	Progression	all wt
74	74	male	FOLFIRI	Bevacizumab	LEFT	Stable disease	KRAS mut (G12V)
75	50	female	FOLFOXIRI	NO	LEFT	Partial response	all wt
76	62	female	FOLFOXIRI	Bevacizumab	LEFT	Stable disease	BRAF mut (V600E)

Table 9. Detailed presentation of the characteristics of the mCRC group

mCRC Patients	**CEA Before**	**CEA Middle**	**CEA After**	**Ca19-9 Before**	**Ca19-9 Middle**	**Ca19-9 After**
22	15,3	2,5	0,5	51	-	16
23	5,54	5,7	-	10	5,5	-
24	118	46	35	<2	<2	<2
25	1,42	2,83	-	9,89	11,37	-
26	1,7	3,36	-	18	7,5	-
27	26,7	214,2	52,9	-	26,11	28,85
28	-	-	-	-	-	-

29	363	115,85	-	-	31,6	-
30	868	64	-	1583	185	-
31	24	-	-	24	24	-
32	1,63	9,97	6,44	-	<2	<2
33	30,21	-	-	71,05	-	-
34	-	-	13	-	-	33
35	32,7	19,41	3,96	28,5	21,73	2,63
36	5,62	28,82	25,2	4,42	<2	<2
37	-	1,34	1,1	-	7,84	4,3
38	2,18	2,81	2,87	4,8	6,61	7,34
39	82,21	34	66,84	68,37	4	5,65
40	12,7	12	1,4	234	30	34
41	9,51	-	-	15,01	-	-
42	5,19	12,4	1,77	241,9	24,06	19,59
43	4,93	3,39	3,5	<2	<2	<2
44	31,28	25,13	72	226,16	284,17	780
45	4,37	5,7	12,25	<2	<2	<2
46	-	-	-	-	-	-
47	26	12	2,7	51	11	-
48	2,47	-	3,53	-	-	6,68
49	355	813	-	224,4		
50	1,3	1,74	2,2	-	13	17
51	1,3	-	1,24	2,03	-	2,6
52	23,27	6,6	-	34,75	29	-
53	5,1	2,13	-	<2	<2	-
54	22,6	3,92	5,3	91,2	57,3	64
55	22,18	14,19	42,3	-	<2	<2
56	1,76	3,4	3,9	<2	<2	-
57	108	30,69	8,3	<4	-	-
58	-	-	-	-	-	-
59	2,5	2,5	-	9	11,46	-
60	263	3	6,22	<2	<2	-
61	90,81	6,9	65,5	-	-	10,5
62	61	8,8	17	77	24	14
63	-	-	-	-	-	-
64	4,2	2,64	2	<2	<2	<2
65	335	156,87	171	75	87,5	-
66	9	12	-	1165	657,4	-
67	5,96	-	-	4,01	-	-
68	1,78	1,6	-	-	17	-
69	7,83	3,5	4,8	26,9	9	-
70	132,6	330	497	<2	<2	<2
71	4	-	2,16	6	-	4,8
72	18,5	15,94	7,97	2,5	3,63	-
73						
74	2,2	2,3	2,1	-	19	19,48
75	1882	134	-	1255	35	-
76	98,6	16,25	-	301	-	-

Table 10. CEA and CA-19.9 levels for mCRC patients before the beginning of their therapy and at the end of it

5.3 MN frequency evaluation

MNf was measured for all patients of our study and their characteristics are presented on Table 5. Counts of MNf were measured at three different time points of therapy (before, middle and after). In Table 11 the descriptive statistics and comparisons of micronuclei (MNf) are shown. There is a statistically significant difference at the initial stage (32.3±9.6) the middle of the therapy (22.0±8.4) and final stage (22.7±9.3) ($F(2, 125)=127.671$, $p<0.001$) when total number of cases were included. When the sample were divided into two parts (laRC and mCRC) Mann-whitney showed a significant effect between the three periods both for laRC ($\chi2(2)=19,500$, $p<0,001$) and mCRC ($\chi2(2)=57,691$, $p<0.001$). Our data from the MN assay on control group, mCRC and laRC patients are presented in Table 12, Table 13 and Table 14 respectively.

	MNf	Mean	SD	F(df1, df2)=	Friedman
Total	**Initial**	32,3	9,6	F(2, 128)=127,671, p<0,001	$\chi^2(2)=75,390$ p<0,001
	Middle	22,0	8,4		
	Final	22,7	9,3		
Non metastatic	**Initial**	29,5	5,7	F(2, 126)=1,304 p=0.275	$\chi^2(2)=19,500$, p<0,001
	Middle	17,7	4,7		
	Final	20,8	5,5		
Metastatic	**Initial**	32,9	10,2		$\chi^2(2)=57,691$, p<0,001
	Middle	23,0	8,8		
	Final	23,2	9,9		

Table 11. Number of micronuclei MNf at three periods (initial, middle and final) time of the therapy.

	Groups	BN cells scored	Mean ± SE	G	P
BNMN	**Control**	25000	7.28 ± 1.06		
	mCRC Before	55000	29.3 ± 9.07	38.00	**<0.001**
	laRC Before	21000	26.4 ± 4.82	30.15	**<0.001**
MN cells	**Control**	25000	8.18 ± 1.11		
	mCRC Before	55000	32.3 ± 9.97	41,1	**<0.001**
	laRC Before	21000	29.6 ± 5.21	33,76	**<0.001**
CBPI ± SE *(Mean ± SE)*	**Control**	1.29 ± 0.03			
	mCRC Before	1.32 ±0.004			
	laRC Before	1.36 ±0.007			

Table 12. Statistical analysis of the mean BNMN and MNf of healthy controls and (A) mCRC/laRC patients at the beginning (Before). Values in bold font indicate statistically significant differences (P<0.05) compared with the controls. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

Based on the absence of a significant difference of the CBPI values between: A) CRC groups and control and B) each sampling point for each response group, we are able to assume that our data is resulted by the cancer itself and the different treatments. Our data indicates that MNf is significantly higher in patients with mCRC or laRC than in healthy individuals (G = 41.1, p<0.0001 and G=33.76, p<0.0001 respectively). Moreover, the relative difference of MNf at the middle of the therapy in relation to that before the beginning of the therapy has been calculated for each patient and the mean values were 31.1±19.0 with median of 33.3 (range from -42.11 to 65.71). Mean values were not significantly different (t-test p=0,130, MW p=0,216) for laRC (36.5±10.7, median 39.3) and mCRC (29.0±21.1, median 32.7). Therefore, there was no significant difference between mCRC and laRC groups (Figure 20).

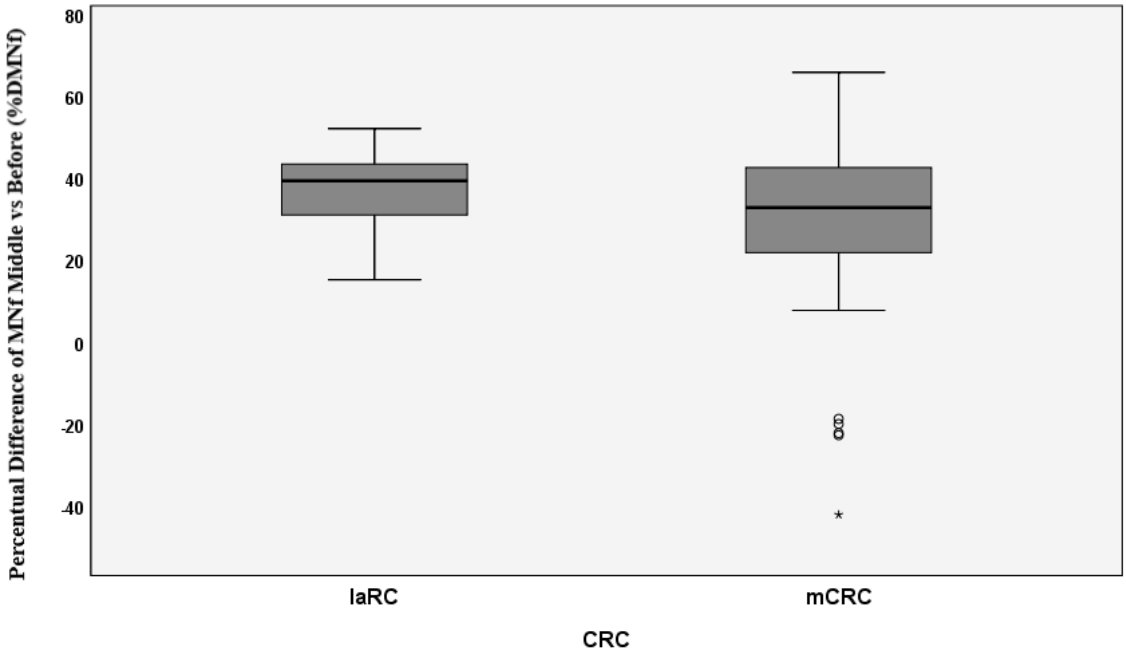

Figure 20. Percentual difference between middle and before measurements of MNf for metastatic colorectal cancer (mCRC) and locally advanced rectal cancer (laRC). * = extreme value as marked in SPSS

MNf analysis for the whole mCRC group revealed that a borderline significance is extracted when the middle of the treatment (Middle) is compared to the beginning of the treatment (Before) (p=0.05), while MNf did not exhibit a further significant decrease at the end point (after) (Table 13). After stratification of patients according to their disease response, a relative pattern of a steady drop of MNf was observed. In detail, even though patients with progressive and stable disease exhibit an

insignificant decrease of their MNf in the middle (G =1.60, p=0.200 and G =3.48, p=0.060 respectively) and at the end of their treatment (G =2.13, p=0.14 and G=3.55, p=0.06 respectively), those with disease progression had lower decrease of MNf than those with stable disease (Table 13). On the contrary, patients with partial response presented a statistically significant decrease of their MNf both at the middle and at the end of the treatment (G=5.16, p=0.02 and G=3.94, p=0.04 respectively) (Table 13). However, since there was only one patient with complete response no statistical analysis of his data was done even though his MNf grossly followed the decreasing trend of those with partial response (before the treatment MNf was 28 at the middle 18 and at the end 15).

mCRC		**Sampling Point**	**BN cells scored**	**Mean ± SE**	**G**	**P**
All patients	**BNMN**	**Before**	55000	29.3 ± 9.07		
		Inter	55000	19.4 ± 6.86	3.90	**0.04**
		After	55000	19.6 ± 8.46	3.73	0.05
	MN cells	**Before**	55000	32.3 ± 9.97		
		Inter	55000	22.2 ± 8.28	3.65	0.05
		After	55000	22.6 ± 9.72	3.35	0.06
	CBPI ± SE *(mean ± SE)*	**Before**	1.32 ± 0.004			
		Inter	1.32 ± 0.004			
		After	1.30 ± 0.002			
Progressive disease	**BNMN**	**Before**	13000	28.8 ± 10.03		
		Inter	13000	21.3 ± 6.3	2.20	0.14
		After	13000	20.9 ± 9.66	2.46	0.11
	MN cells	**Before**	13000	31.3 ± 10.76		
		Inter	13000	24.6 ± 10.93	1.60	0.20
		After	13000	23.6 ± 10.92	2.13	0.14
	CBPI ± SE *(mean ± SE)*	**Before**	1.29 ± 0.001			
		Inter	1.32 ± 0.004			
		After	1.31 ± 0.003			
Stable disease	**BNMN**	**Before**	18000	30,2 ± 8,38		
		Inter	18000	20,3 ± 6,4	3.77	0.05
		After	18000	20.1 ± 8.77	3.93	**0.04**
	MN cells	**Before**	18000	33.1 ± 9.35		
		Inter	18000	23.1 ± 7.39	3.48	0.06

		After	18000	23 ± 10.02	3.55	0.06
	CBPI ± SE *(mean ± SE)*	**Before**	1.32 ± 0.003			
		Inter	1.31 ± 0.002			
		After	1.30 ± 0.001			
Partial Response	**BNMN**	**Before**	23000	29.0 ± 9.54		
		Inter	23000	17.8 ± 5.98	5.15	**0.02**
		After	23000	18.7 ± 7.88	4.29	**0.03**
	MN cells	**Before**	23000	32,5 ± 10.51		
		Inter	23000	20.6 ± 7.4	5.16	**0.02**
		After	23000	22.0 ± 9.30	3.94	**0.04**
	CBPI ± SE *(mean ± SE)*	**Before**	1.33 ± 0.006			
		Inter	1.31 ± 0.005			
		After	1.30 ± 0.001			

Table 13. Statistical analysis of the mean BNMN and MNf of mCRC patients at the beginning (Before), the middle (Inter) and at the end (After) of the treatment for all mCRC patients and according to their disease response (progressive disease, stable disease, partial response and complete response). Values in bold font indicate statistically significant differences (P<0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

Regarding the laRC group, since our primary objective was to evaluate our biomarkers' prognostic value of therapies response, we tested for MNf after laRC surgery in approximately half of our patients (12 out of 21). Therefore, a difference in counted BN cells is observed for the sampling point "surgery" relative to the other two sampling points (Table 14). However, our data indicates a similar decreasing trend as observed in the mCRC group. In detail, a significant decrease of MNf (G=4.01, p=0.04) is found at the end of the treatment (sampling point "after") in relation to the beginning (Table 14). However, even though patients maintain lower MNf than what they had before treatment, a slight increase is observed after surgery (G=3.00, p=0.08) (Table 14). When divided in subgroups our data indicates a steady decrease of MNf that positively correlates to the disease response. Patients with progressive disease present a lower decrease of MNf after treatment (p=0.24), than those patients with stable disease (G=2.82, p=0.09), partial response (G=4.50, p=0.03) or complete response (G=4.77 p=0.02) (Table 14).

laRC		Sampling Point	BN cells scored	Mean ± SE	G	P
All patients	**BNMN**	**Before**	21000	26.4 ± 4.82		
		After	21000	16.9 ± 4.00	4,01	**0.04**
		Surgery	12000	17.4 ± 3.57	3.57	0.06
	MN cells	**Before**	21000	29.6 ± 5.2		
		After	21000	19.7 ± 4.7	3.85	**0.04**
		Surgery	12000	20.8 ± 5.48	3.00	0.08
	CBPI ± SE *(mean ± SE)*	**Before**	1.36 ± 0.007			
		After	1.32 ± 0.003			
		Surgery	1.27 ± 0.003			
Progressive disease	**BNMN**	**Before**	2000	27.5 ± 6.36		
		After	2000	21.5 ± 4.95	1.45	0.22
		Surgery	1000	16.0 ± 5.1	5.80	**0.01**
	MN cells	**Before**	2000	31.3 ± 10.76		
		After	2000	23.5 ± 6.36	1.35	0.24
		Surgery	1000	17.0 ± 5.2	6.42	**0.01**
	CBPI ± SE *(mean ± SE)*	**Before**	1.30 ± 0.001			
		After	1.40 ± 0.005			
		Surgery	1.26 ± 0.002			
Stable disease	**BNMN**	**Before**	5000	28.7 ± 4.72		
		After	5000	19.7 ± 3.25	3.25	0.07
		Surgery	3000	24.0 ± 4.24	0.83	0.36
	MN cells	**Before**	5000	32.7 ± 4.62		
		After	5000	23.7 ± 4.72	2.82	0.09
		Surgery	3000	27.0 ± 2.83	1.09	0.29
	CBPI ± SE *(mean ± SE)*	**Before**	1.30 ± 0.001			
		After	1.40 ± 0.005			
		Surgery	1.26 ± 0.002			
Partial	**BNMN**	**Before**	11000	24.8 ± 4.14		

Response		**After**	11000	14.9 ± 2.93	4.71	**0.02**
		Surgery	6000	14.4 ± 2.64	5.25	**0.02**
		Before	11000	27.8 ± 4.30		
		After	11000	17.5 ± 3.18	4.50	**0.03**
	MN cells	**Surgery**	6000	17.9 ± 3.18	4.14	**0.04**
	CBPI ± SE	**Before**	1.35 ± 0.008			
	(mean ± SE)	**After**	1.30 ± 0.001			
		Surgery	1.30 ± 0.010			
Complete response	**BNMN**	**Before**	3000	30.0 ± 6.25		
		After	3000	18.7 ± 5.50	5.05	**0.02**
		Surgery	2000	22.0 ± 4.24	2.41	0.11
		Before	3000	33.7 ± 7.37		
		After	3000	22.0 ± 6.08	4.77	**0.02**
	MN cells	**Surgery**	2000	22.0 ± 4.24	4.77	**0.02**
	CBPI ± SE	**Before**	1.45 ± 0.001			
	(mean ± SE)	**After**	1.32 ± 0.001			
		Surgery	1.22 ± 0.001			

Table 14. Statistical analysis of the mean BNMN and MNf of laRC patients at the beginning (Before), the end of the treatment (After) and after surgery (Surgery) for all laRC patients and according to their disease response (progressive disease, stable disease, partial response and complete response). Values in bold font indicate statistically significant differences ($P<0.05$) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

5.4 Evaluation of MNf as a prognostic biomarker

The prognostic significance of MNf in mCRC and laRC patients was roughly established using ROC curve analysis. Variation of MNf expressed as %DMNf between initial and middle measurements was estimated by setting binary outcome variables for two scenarios: Progressive disease vs stable/partial/complete response (scenario 1) and progressive/stable disease vs partial/complete response (scenario 2). For scenario 1, the best set of sensitivity and specificity was found at 29% difference between middle and initial MNf measurements (sensitivity 36% and specificity 87.0%). While the highest specificity (87.2%) was achieved at 31% reduction of MNf. For scenario 2, the best set of sensitivity and specificity was found again at 29%

difference between middle and initial MNf measurements (sensitivity 72.7% and specificity 59.3%) and the highest specificity (59.6%) was found for 31% reduction of MNf (Table 15).

	Outcome							
	Prog. (N_T=15)		**S.D. / P.R. /C.R. (N_T=61)**		**Prog. / S.D. (N_T=38)**		**P.R. / C.R. (N_T=38)**	
Limit	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
≤33%	9	25,0%	27	75,0%	20	55,6%	16	44,4%
>33%	6	15,0%	34	85,0%	18	45,0%	22	55,0%
≤ 31%	9	31,0%	20	69,0%	19	65,5%	10	34,5%
>31%	6	12,8%	41	87,2%	19	40,4%	28	59,6%
≤ 29%	8	36,4%	14	63,6%	16	72,7%	6	27,3%
>29%	7	13,0%	47	87,0%	22	40,7%	32	59,3%

Table 15. Sensitivity and specificity values for three different cutoff points of percentage difference of MNf. It can be seen that when the outcome is progressive disease vs stable/ partial/complete response the best set of sensitivity-specificity is found at 29% difference between middle and initial MNf measurements (sensitivity 36.4% and specificity 87.0%). The highest specificity (87.2%) was found for 31% reduction of MNf. When the outcome was set between stable/progressive disease vs partial/complete response the best set of sensitivity and specificity variables was found for 29% difference (sensitivity 72.7% and specificity 59.3%). NT= Total number of patients, Prog.= progression, S.D.= stable disease, P.R.= Partial response, C.R.= complete response

5.5 Telomerase activity (TA)

As presented in Table 16, based on the non-parametric analysis (Kruskal-Wallis) there is no significant difference between patients' mean TA before the beginning of their therapy (p=0.256) [progressive disease: 2.1±1.6 (95% CI: 0.4-3.8), stable disease: 1,4±1,7 (95% CI: 0,2-2,6), partial response: 0,8±1,0 (95% CI:0,1-1,5), complete response: 0,6±0,6 (95% CI:-5,2 - 6,4)], at the middle sampling point (p=0.072) [progressive disease: 2,8±0,8 (95%CI:1,9-3,7), stable disease: 1,5±1,3 (95%CI:0,5-2,4), partial response: 1,1 ±1,4 (95%CI:0,1-2,1), complete response: 0,2±0,1 (95%CI: -1,0-1,4)] or at the third sampling point (p=0.096) [progressive disease: 2,5±1,3 (95%CI: 1,1-3,9), stable disease: 1,1±1,2 (95%CI:0,2-2,0), partial response: 1,1±1,3 (95%CI: 0,2-2,0), complete response: 0,9±1,2 (95%CI: -10,2-12,0)]. However, as presented in Table 16 and Figures 21/22, Mann-Whitney test and t-test proved that the mean of patients who eventually developed progressive disease, exhibited an overall higher level of TA in relation to all other response groups before the beginning of the

therapy (p=0.012 and p=0.009 respectively). Thereafter, TA was increased and remained elevated during the middle and third sampling points respectively. In Figure 4 the comparison of patients with progressive CRC (mCRC and laRC) against all other patients (stable disease, partial and complete response) exhibited a significantly statistical difference (t-test p=0.009, Mann-Whitney p=0,012).

Telomerase Activity		n	**Mean**	**SD**	**95%**		**Minimum**	**Maximum**	**p**
					LL	**UL**			
Before	**Progression**	6	2,1	1,6	0,4	3,8	0,1	3,7	0.256
	Stable disease	10	1,4	1,7	0,2	2,6	0,0	3,8	
	Partial response	10	0,8	1,0	0,1	1,5	0,0	2,4	
	Complete Response	2	0,6	0,6	-5,2	6,4	0,2	1,1	
	Total	28	1,3	1,4	0,7	1,8	0,0	3,8	
Middle	**Progression**	6	2,8	0,8	1,9	3,7	1,7	3,7	0.072
	Stable disease	10	1,5	1,3	0,5	2,4	0,0	3,5	
	Partial response	10	1,1	1,4	0,1	2,1	0,0	3,2	
	Complete Response	2	0,2	0,1	-1,0	1,4	0,1	0,3	
	Total	28	1,5	1,4	1,0	2,1	0,0	3,7	
After	**Progression**	6	2,5	1,3	1,1	3,9	0,1	3,6	0.096
	Stable disease	10	1,1	1,2	0,2	2,0	0,0	3,3	
	Partial response	10	1,1	1,3	0,2	2,0	0,0	3,3	
	Complete Response	2	0,9	1,2	-10,2	12,0	0,0	1,8	
	Total	28	1,4	1,3	0,9	1,9	0,0	3,6	

Table 16. Telomerase activity measured at 3 time points, before (before the initiation of the therapy), middle (at the middle of therapy for mCRC and at the end of therapy for laRC) and after (at the end of the therapy for mCRC and after surgery for laRC) is presented for all CRC cases. p-values were estimated with Kruskal-Wallis test. n= Number of patients

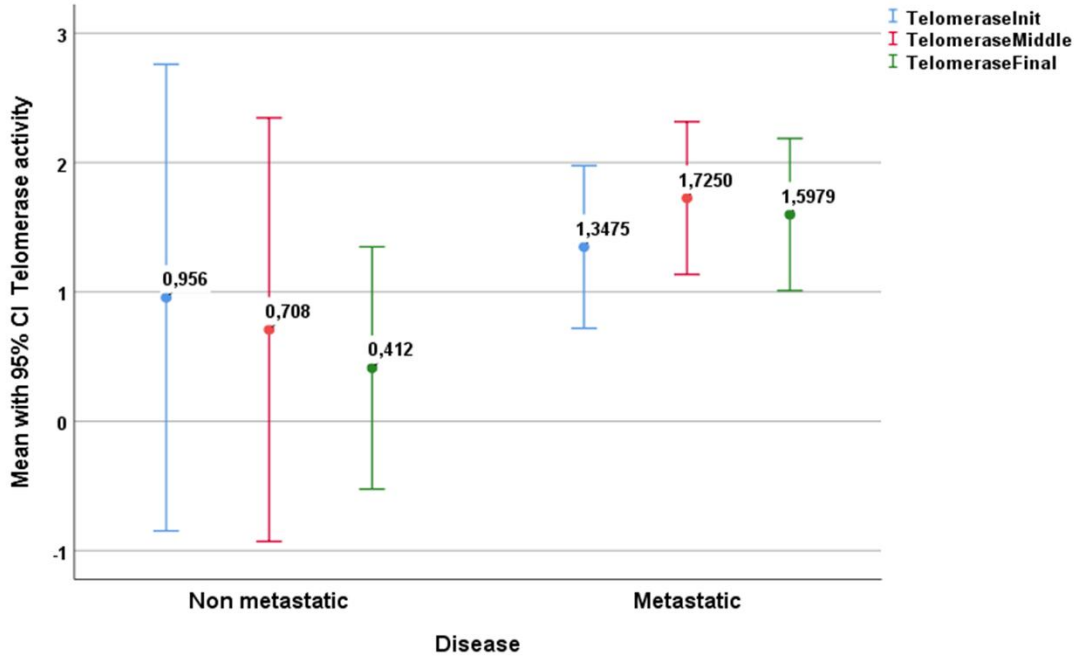

Figure 21. Mean values with 95% confidence intervals (C.I.) of telomerase activity for initial, middle and final sampling points

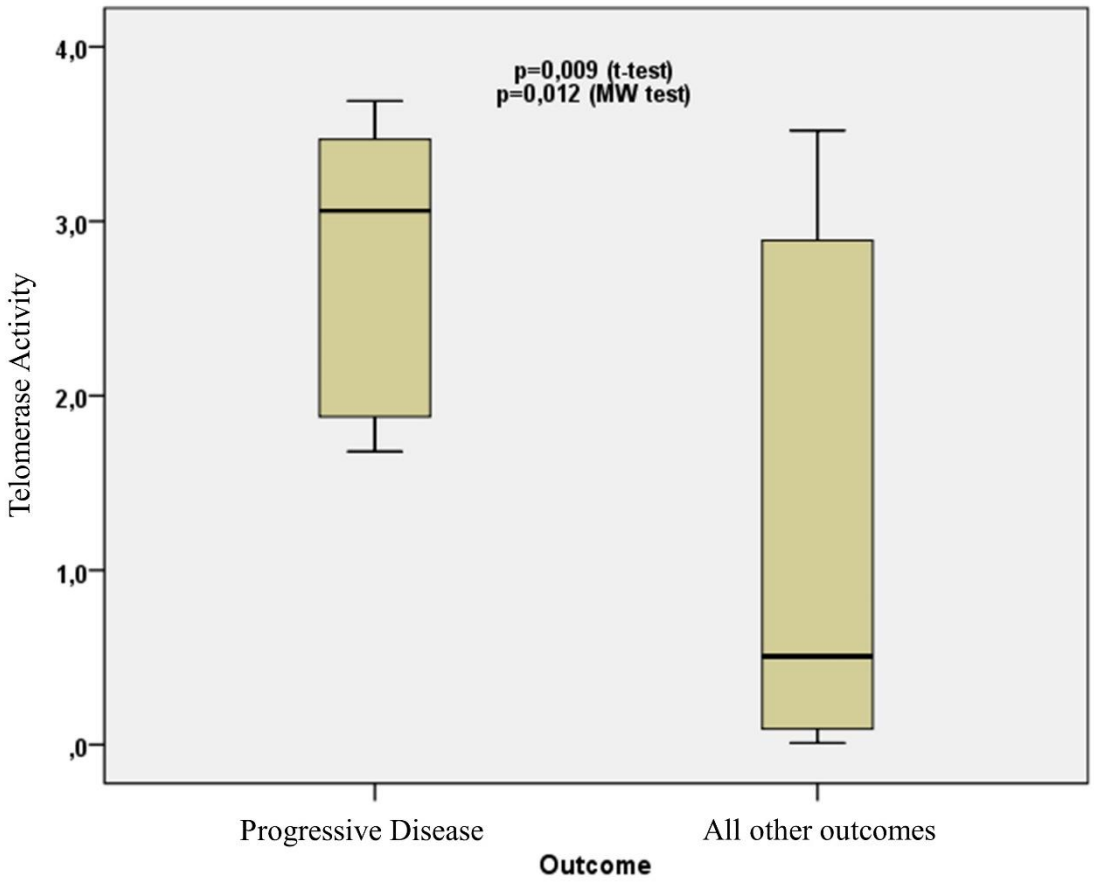

Figure 22. Comparison of patients with progressive CRC (mCRC and laRC) against all other patients (stable disease, partial and complete response) exhibited a significantly statistical difference (t-test p=0.009, Mann-Whitney p=0,012)

5.6 Correlation between MNf and TA with CEA and CA 19.9 levels in mCRC and laRC patients

This doctoral thesis had set the correlation between MNf and TA with CEA and CA 19.9 levels in mCRC and laRC patients as its secondary objective. However, due the fact that CEA and CA-19.9 levels were evaluated as part of the clinical management of each patient in the Department of Clinical Oncology, not only the sampling points would differ from those of MNf/TA (in many cases by a month) but also a great number of samples were not taken. Therefore, in order to avoid methodological errors the correlation was not performed. Tables 8 and 10 present the levels of CEA and CA-19.9 that were chronologically closer to MNf and TA sampling points.

6. Discussion

Colorectal cancer is and, according to the latest statistics, will be one of the leading causes of cancer-related morbidity and mortality worldwide for both sexes. Due to the delayed onset of symptoms, poor patient compliance with the various diagnostic modalities (namely colonoscopy) and the relatively low suspicion of the general public, a great number of patients are presented with metastatic or advanced CRC. However, CRC patients with distant metastases are characterized by the worst prognosis since a significant number of them develops resistance to their therapy at some point of its course. Unfortunately, diagnosis of chemoresistance is most often delayed, allowing for cancer progression to take place before these patients receive second or third line treatments. At the meantime, patients and their relatives are struggling with the various therapy-related side effects while at the same time health care systems are dealing with immense costs of both the ineffective therapies and the prolonged hospital stay. Therefore, the need for early and reliable recognition of chemoresistance has become greater than ever. In order to fulfill this need for identification of effective prognostic and predictive biomarkers in CRC, this doctoral thesis focused on unveiling possible prognostic values of two novel biomarkers (MNf and TA) in PBLs isolated from patients with mCRC and laRC blood samples. Although the findings are hypothesis driving, given the relatively small number of patients tested, the generated data provides further proof that MNf is not only significantly increased in CRC but it could also serve as a promising biomonitoring tool for mCRC and laRC prognosis. More specifically, according to the data generated here, patients diagnosed with mCRC and laRC, regardless of age, sex, BMI and genetic status had high rates of BNMN and MNf. This was found both before and throughout the systemic therapy, even though a declining trend was evidenced after the beginning of the therapy. Nevertheless, even if MNf decreased, it never reached the MNf of healthy individuals.

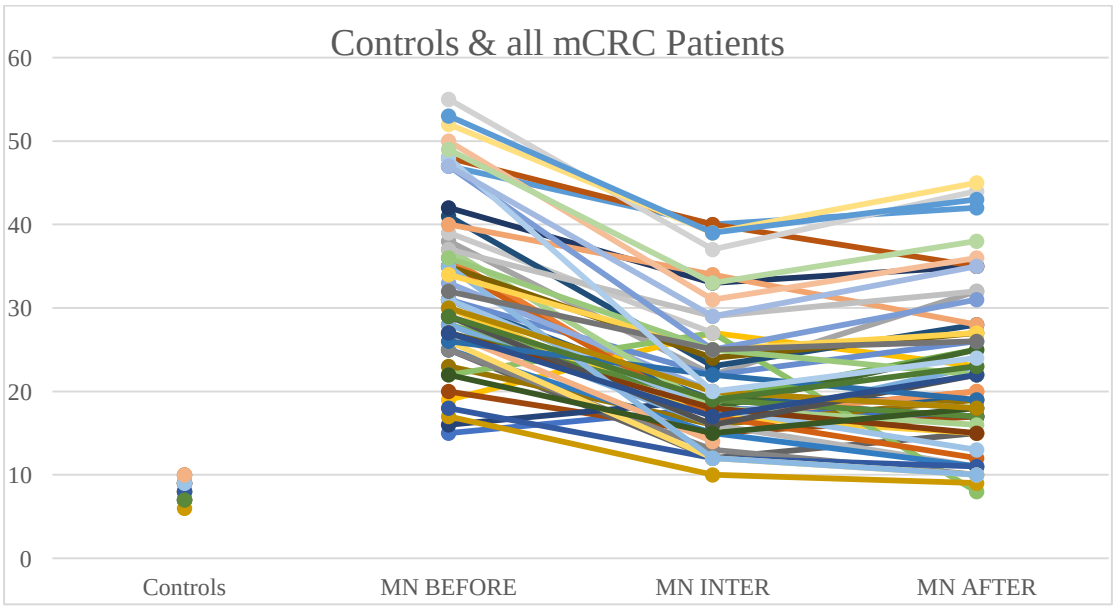

Figure 23. Graphic representation of MNf in controls and the trends between sampling points for all mCRC patients

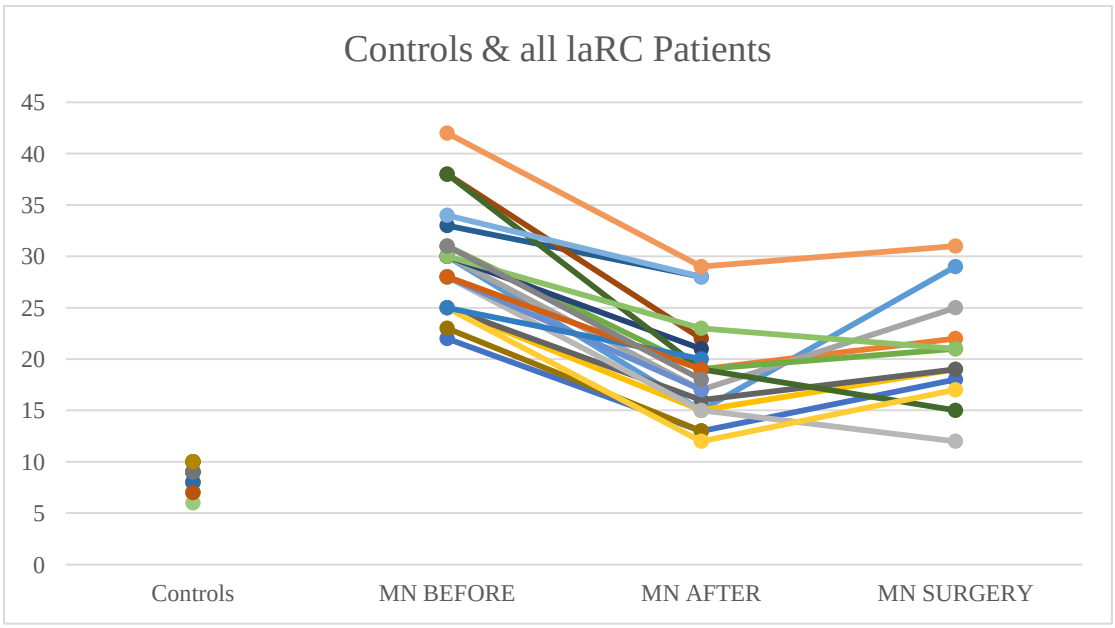

Figure 24. Graphic representation of MNf in controls and the trends between sampling points for all laRC patients

In parallel, both mCRC and laRC patients had the same CBPI values with that of healthy individuals, that remained stable across all time points regardless the therapy or the response. We can speculate that the observed decrease in the mean MNf after the first trimester of the therapy (middle sampling point) reflects the response of the organism to the treatment accomplished by the depletion of the sensitive cancerous "background". Figures 23-27 illustrates how MNf may follow a steep decreasing trend in patients with partial/complete response or a shallow decrease in patients with disease progression.

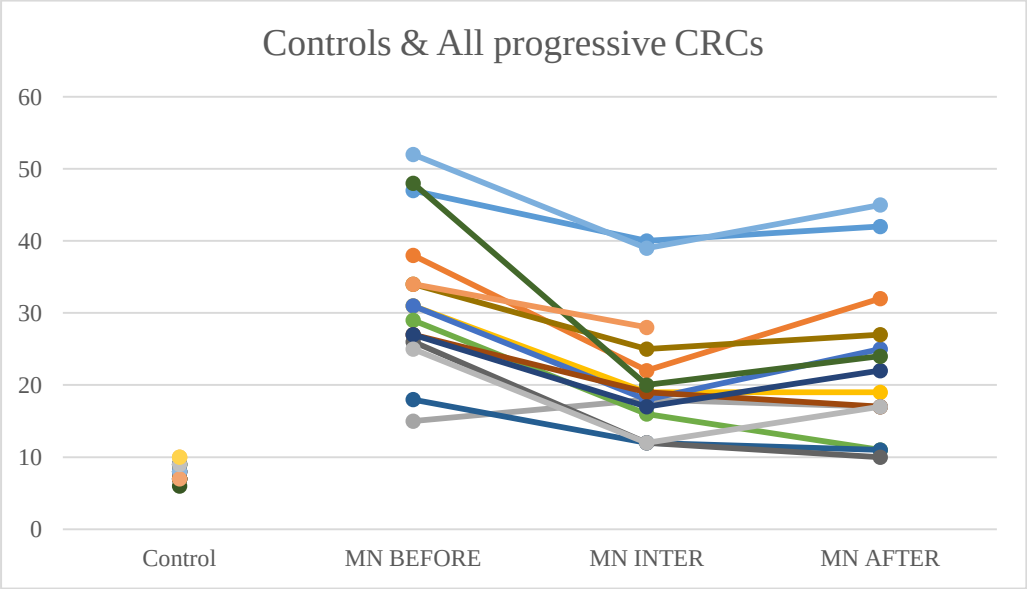

Figure 25. Graphic representation of MNf in controls and the trends between sampling points for all patients (mCRC and laRC) with progressive disease

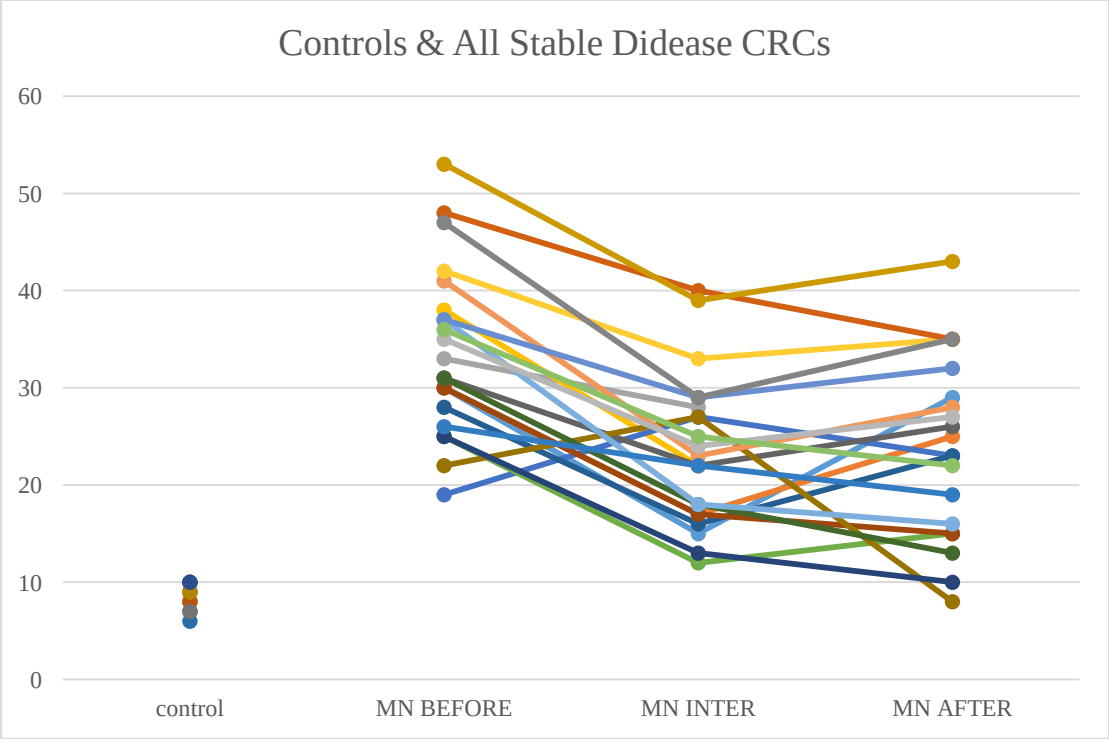

Figure 26. Graphic representation of MNf in controls and the trends between sampling points for all patients (mCRC and laRC) with stable disease

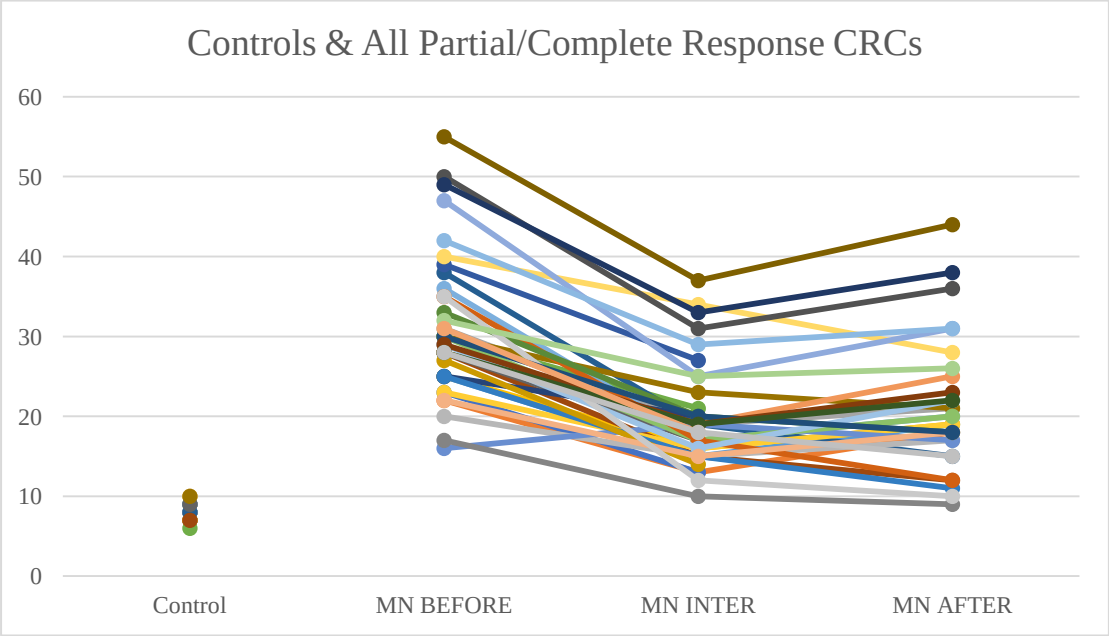

Figure 27. Graphic representation of MNf in controls and the trends between sampling points for all patients (mCRC and laRC) with partial or complete response

A possible explanation of our findings lies in the fact that MN assay is a sensitive indicator of genomic damages of exogenous and endogenous origin (192) while MNf in PBLs, despite the unknown underlying mechanism, is shown to positively correlate with chromosomal and genomic instability, which is one of the pillars in colorectal carcinogenesis. Therefore, the emergence of a resistant cluster of cancer-cells against the sensitive "background" can be indirectly identified through MNf. However, the following increase of mean MNf (but never to the degree before treatment) raises a challenge for its clinical interpretation. The first scenario is that of a 'gradual emergence of a resistant clone'. First, sensitive cancer cells are depleted and so MNf and BNMN decrease. Subsequently, resistant ones emerge as they do not have to compete for energy or oxygen supply. In fact, this scenario could explain the fluctuations in MNf observed in the 3 response groups during the therapy. At the middle of therapy, both the good and no response groups began killing sensitive cells and decreased their MNf numbers, while the stable group did not. While the good responders then continued to deplete sensitive cancer cells, the non-responders began to increase cancer cells and their MNf increases accordingly, while the stable group maintained almost the same cell number and MNf. The second scenario is the 'long-term chemo-effect'. As mentioned before, both oxaliplatin and irinotecan increase MNf. However, in order to do that, certain time is needed. This is why, even though cancer load decreases and the drug accumulation is not yet at its peak, the MNf also decreases. However, as the rate of cancer cells decrease diminishes and the accumulation of the drug reaches its peak, the MN-increasing properties of oxaliplatin and irinotecan become evident. This scenario can also explain why at the end of the treatment the partial/complete response group did not differ significantly, in terms of mean BNMN and MNf, than the other two groups (progressive and stable disease). Interestingly though, even if the majority of the patients exhibited a 'v'-shaped trend of MNf (decrease and then slight increase), there were 4 patients who exhibited a reverse 'v'-shaped trend, with an increase of mean MNf at 3 months, and a

subsequent decrease at 6 months of treatment despite favorable disease response, as shown in Figures 23/24. In fact, these 4 patients who presented reverse ‘v’-shaped trend, were treated with a combined therapy of FOLFIRI plus any biologic agent. Therefore, MNf may not be appropriate to be used as prognostic biomarker in such patients, and special caution is suggested for its use. Nonetheless, it could be used as a biomonitoring tool of cancer load.

However, in order to estimate whether MNf could serve as prognostic biomarker, a validation of its sensitivity and specificity in identifying progressive and stable disease from partial or complete response was carried. Therefore, the present thesis identified that a decrease of MNf less than 29% between middle and initial MNf measurements can discriminate between progressive disease from stable/responsive disease with sensitivity 36% and specificity 87.0%, while if the threshold is set at 31% reduction of MNf then specificity reaches its highest value (87.2%). On the other hand if the threshold of decrease is set at 29% for discrimination between stable/progressive and responsive disease then sensitivity reaches 72.7% and specificity 59.3%. These findings are important because early identification of those patients who are more likely to develop progressive disease can allow clinicians to take early decisions on therapy selection.

As for TA, even though the number of patients to which TA was evaluated is rather small, our results indicate its potential in CRC prognosis suggesting the need for future studies with greater patient sets. Despite the fact that statistical analysis between each response group did not reach statistical significance, our data indicate that TA is relatively higher in patients with progressive disease than those with partial or complete response at all sampling points. Moreover, in patients with progressive disease, an increase of TA is observed in the middle of their therapy, suggesting that patients who are more likely to develop progressive disease are more likely to have upregulated TA at the middle of their therapy. This can be explained by numerous studies that have identified telomerase as a key target of multiple carcinogenic pathways such as the PI3K/AKT/mTOR, the RAS/RAF/MEK/ERK 1/2, the JAK/STAT and the JAK/PI3K/AKT/HSP90/mTORC1 (193). An interesting finding highlighting the complexity of hTERT regulation is that EGFR-mediated MAPK signaling attenuates Groucho-mediated gene repression, establishing a node for crosstalk between the EGFR, Notch, WNT, and TGF-β signaling pathways (193). A graphical presentation of the aforementioned mechanisms is shown in Figure 28. Unfortunately, since our primary objective was to investigate possible prognostic significance of these biomarkers, we did not examine any de-regulation of the aforementioned pathways.

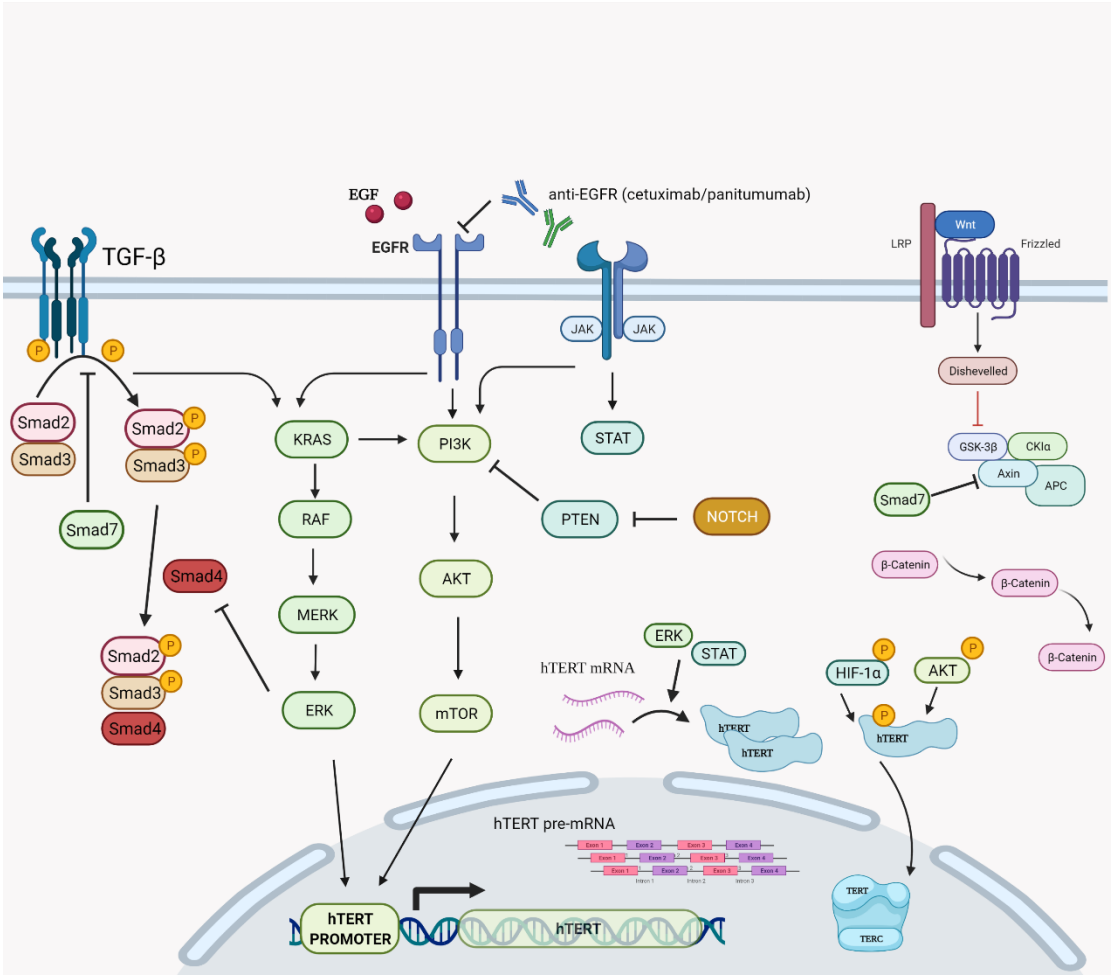

Figure 28. Graphical presentation of the various cellular cascades implicated in hTERT expression, telomerase activity and telomeres length. During transcription of hTERT gene, positive regulation is exerted via the PI3K/AKT/mTOR, the RAS/RAF/MEK/ERK 1/2 and the JAK/STAT pathways primarily on hTERT's promoter. Another step of positive regulation is found during the translation and post translation phase of hTERT mRNA since the RAS/RAF/MEK/ERK pathway is described to regulate hTERT mRNA translation while the PI3K/AKT/mTOR pathway seems to be involved post-translationaly via direct phosphorylation of hTERT by AKT and HIF-1α. Rat sarcoma (RAS), Rapidly Accelerated Fibrosarcoma (RAF), Mitogen-activated protein kinase kinase (MEK also known as MAP2K, MAPKK), extracellular signal-regulated kinase (ERK, also known as MAPK), phosphatidylinositol 3-kinase (PI3K), Protein kinase B (PKB, also known as Akt), mechanistic target of rapamycin kinase (mTOR), hypoxia-inducible factor-1α (HIF-1α). Created with BioRender.com

Finally, in order to thoroughly understand and interpret these results, it is important to know how these biomarkers are affected by the different therapeutic agents used in our study. To begin with, FOLFOX, FOLFIRI and FOLFOXIRI regimens are the three principal first-line therapeutic regimens administered for stage IV CRC according to HeSMO guidelines. They share two components, folinic acid (FA) and 5-fluorouracil (5-FU), while oxaliplatin and irinotecan are the compounds that differentiate the first two respectively or if combined make the later. CAPOX on the other hand, which along with FOLFOX is primarily used to treat laCRC, contains

capecitabine and oxaliplatin. Based on bibliographic evidence validating the effect of these agents upon MNf and TA, it is evident that there is no universal effect. Overall it is reported that 5-FU, capecitabine, oxaliplatin and irinotecan increase BNMN, while FA significantly reduces it (194). Interestingly, as mentioned earlier, our data provides further proof that irinotecan and therefore FOLFIRI may increase MNf. However, for the most part, little data exists regarding any alterations of TA concomitant to administration of these substances. According to Akiyama et al, TA was decreased in human haematopoietic cancer cell lines, Daudi and U937, treated with irinotecan (195). However, there is no data suggesting any possible alterations of TA in CRC cell lines. Chung et al, using the HCT116 and DLD1 CRC cell lines demonstrated a decreased TA concurrent to 5-FU administration possibly via by STAT3 inhibition (a potent activator of hTERT promoter). However, despite thorough research in various databases, to date there is no study available evaluating the effect of the chemotherapeutic regimens of FOLFOX, FOLFIRI, FOLFOXIRI or CAPOX on any of the biomarkers-in study. Given the great number of our patients treated with some kind of biologic agent (bevacizumab, cetuximab, panitumumab or aflibercept), we expanded our research to include them as well. The only published data focusing on MNf comes from an in vivo cytogenetic assay performed in male Wistar rats, where cetuximab did not elicit any genotoxic effects (196). However, the results should be considered of limited value due to the lack of immunoreactivity of Cetuximab with rat tissues.

7. Conclusions

Despite critical advances in most aspects of CRC management, it is indisputably one of the most important burdens of global health due to the related increased morbidity and mortality. Metastatic CRC, the final stage of CRC, remains a true challenge, not only for researchers and clinicians, but also for the socioeconomic system. This is because its inherent biologic complexity and diversity makes it difficult to implement a universal approach in designing effective therapeutic and study protocols. On the contrary, even though laRC is a rather favorable type of CRC due to the absence of distant metastases, there still is a metastatic potential. Therefore, early recognition of chemoresistance is crucial. This doctoral thesis made it possible not only to recognize possible prognostic significance at an early stage of therapeutic management for two novel biomarkers (MNf and TA), but also to suggest a relative threshold for MNf (decrease of MNf less than 29% between middle and initial MNf measurements can discriminate between progressive disease from stable/responsive disease with sensitivity 36% and specificity 87.0%, while at 29% decrease discrimination between stable/progressive and responsive disease is able with sensitivity of 72.7% and specificity 59.3%) as a discrimination point between progressive and stable/responsive disease. On the other hand, even if TA did not prove to have significant correlation with disease response, possibly due to the small number of patients, it was exhibited that patients with progressive disease had higher mean TA than all other patients suggesting that further analysis in greater patient sets is needed. In a nutshell, the results of the current study should be interpreted with caution due to the limitations of the protocol (relatively small number of cases, different systemic treatment, different mutations subtypes, not randomized manner etc.) and could mainly serve as a hypothesis driving study for further evaluation in the future.

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Effect of systemic treatment on the micronuclei frequency in the peripheral blood of patients with metastatic colorectal cancer

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Abstract. Colorectal cancer (CRC) is the third most diagnosed type of cancer affecting males, and the second most diagnosed type of cancer affecting females, and one of the leading causes of cancer-related mortality globally. The estimation of the micronuclei (MN) frequency in peripheral blood lymphocytes (PBLs) from patients with CRC is proposed as a prognostic/predictive easy-to-use biomarker. In this study, we aimed to investigate the effects of systemic treatment on the MN frequency in PBLs from patients with CRC in order to determine the effectiveness of the MN frequency as a biomarker. For this purpose, from 2016 to 2018, we quantified the MN frequency as a prognostic/predictive biomarker in serial samples from 25 patients with metastatic CRC (mCRC) using cytokinesis block micronucleus assay (CBMN assay). The MN frequency in the PBLs of the patients was evaluated before, during the middle and at the end of the therapy (approximately 0, 3 and 6 months). The results revealed a common pattern regarding the fluctuation in the MN frequency. Statistical analysis confirmed that when the disease response was estimated with radiological criteria, a good response was depicted at the MN frequency and vice versa. Consequently, the findings of this study suggest that the MN frequency may serve as a promising prognostic/predictive biomarker for the monitoring of the treatment response of patients with CRC.

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Abbreviations: CRC, colorectal cancer; MN, micronuclei; MNf, micronuclei frequency; CBMN assay, cytokinesis block micronucleus assay; CIN, chromosomal instability; MSI, microsatellite instability; CIMP, CpG island methylation phenotype; LCC, left-sided colon cancer; RCC, right-sided colon cancer; mCRC, metastatic colorectal cancer; BN cells, binucleated cells; CBPI, cytokinesis block proliferation index; BNMN, binucleated cells with micronuclei; FOLFIRI, folinic acid with 5-fluorouracil and irinotecan; FOLFOX, folinic acid with 5-fluorouracil and oxaliplatin; PBLs, peripheral blood lymphocytes

Key words: metastatic colorectal cancer, micronucleus, prognostic biomarker, predictive biomarker, micronuclei frequency, cytokinesis block micronucleus assay

Introduction

Colorectal cancer (CRC) is third most commonly diagnosed type of cancer affecting males, following lung and prostate cancer, and the second one affecting females, following breast cancer (1). In fact, CRC is a polygenic disease, which arises both from epigenetic, as well as genetic alterations in a variety of oncogenes, tumor suppressor genes, mismatch repair genes and cell cycle regulating genes in colon mucosal cells (2). Due to late diagnosis, approximately a quarter (20-25%) of CRC cases at the time of diagnosis present distant metastases, and another quarter of patients with early resectable CRC will eventually develop metastatic disease, most often in the liver.

It has been described that different pathways lead to carcinogenesis in the colonic epithelium; however, the majors ones are the following: Chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylation phenotype (CIMP) (3). All these pathways attribute to the transformation of an adenoma to carcinoma, a multistep carcinogenic process known as the adenoma-carcinoma sequence (4), which is considered to be a common process in all CRCs (5). As CRC is an heterogeneous disease, it exhibits various clinical manifestations, biological behavior and an in-tumor variety of mutations (6), making it a true

challenge for the clinician. Despite the fact that left-sided colon cancer (LCC) accounts for the majority of CRC cases, the number of cases with right-sided colon cancer (RCCs) is constantly rising (7). The female sex, age, a previous history of cancer and insulin resistance are some of the risk factors that have been associated with RCC, while a low-fiber diet, smoking and alcoholism have been associated with LCC (8,9). Moreover, LCC is commonly associated with metastasis to the liver and lungs, while RCC tends to be more differentiated and is associated with metastasis to the regional lymph nodes and the peritoneal cavity (10). In fact, it is estimated that approximately 22% of patients with CRC present with stage IV metastatic CRC (mCRC) at the time of diagnosis, indicating that, if treated, the expected 5-year-survival rate is only 13% (11). In this setting, chemotherapy is mainly used as a palliative measure in order to improve the quality of life and achieve the optimum survival. However, not all patients with stage IV disease exhibit the same response to treatment, even if the underlying genetic status is the same (12). This is the cornerstone of the research for prognostic and predictive biomarkers; the in-group difference.

Micronuclei (MN), or Howell-Jolly bodies, are small intracellular particles enwrapped in a nuclear envelope. They are formed as a result of acentric chromatid/chromosome fragments (mainly due to extensive DNA damage) or whole chromatids/chromosomes (mainly due to mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation and defects in the cell cycle control system) that during the anaphase of dividing cells do not follow the rest of the chromosomes and are not included in the nucleus during telophase. Instead, enwrapped by the nuclear membrane, they form daughter nuclei-like structures that are just a fraction of the size of the mother nucleus (13,14). Numerous studies have evaluated the use of MN frequency (MNf) in different cell types and lines in order to determine whether it can be used as an effective biomarker for various types of cancer (including lung, bladder and colorectal cancer) (15-17). Almost all of these studies agree that MNf is a sensitive indicator of cancer since, compared to healthy controls, there is a significant increase in MN formation regardless of the type of cancer. However, for patient's convenience, peripheral blood lymphocytes (PBLs) are preferably used. What is more, those who evaluated MNf in CRC did prove an increased MNf (thus indicating its possible use as a diagnostic biomarker), but did not evaluate their patients in the long-term and did not include cases with metastatic disease. Hence, the importance of MNf as a prognostic and/or predictive biomarker in mCRC has not yet been investigated in detail, at least to the best of our knowledge. The only published attempt to illuminate the prognostic properties of MNf, to the best of our knowledge, comes from a team which evaluated MNf in urothelial cells of patients with bladder cancer (18,19).

Thus, under this scope, the present study aimed to assess the efficiency of MNf as a biomarker for the prognosis and disease/treatment prediction of patients with mCRC.

Patients and methods

Patients and study protocol. The protocol of this study was approved by the Ethics Committee for Patients and Biological Material of the University Hospital of Heraklion (Heraklion, Greece). During the period between December, 2016 and February, 2018, 27 patients referred to the Department of Medical Oncology of the University Hospital of Heraklion were enrolled in this study. All patients signed a written consent. The inclusion criteria were as follows: i) Patients with mCRC treated with 1st line systemic treatment according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (20); and ii) an age between 50-75 years. The exclusion criteria were as follows: i) Failure to complete the therapeutic regimen for any reason (toxicity, refusal of the patient, or death); and ii) the refuse of the patient to attend the study. Based on the chemotherapeutic protocol that was selected [folinic acid with5-fluorouracil and oxaliplatin (FOLFOX) or folinic acid with5-fluorouracil and irinotecan (FOLFIRI) with or without a biological factor], patients were further divided into subgroups. Another division of the patients was made based on their body mass index (BMI) before treatment (BMI <25, BMI ≥25 but ≤30, and BMI >30). The RECIST criteria version 1.1 were used for the evaluation of the treatment response (21). According to these criteria, patients were evaluated at the end of the therapy and were divided into 3 subgroups as follows: Good response, stable disease and no response. Peripheral blood samples were collected at fixed time-points, namely before the beginning of the therapy, 3 months after the initiation of treatment and at the end of treatment (at 0, 3 and 6 months of treatment, respectively) for the evaluation for MNf using the cytokinesis block micronucleus assay (CBMN assay). By June, 2018, 25 out of the 27 patients had completed the study. One patient presented with increased toxicity and terminated the therapy and the other one died due to a heart failure as a result of a lower respiratory tract infection. Finally, 10 healthy individuals (5 male and 5 female) were recruited from the Health Center of Agia Varvara, Heraklion, Crete, after receiving a thorough explanation about the study, how their samples would be handled and signing a written consent. The inclusion criteria were an age between 55 and 70 years and a personal history free of cancer, autoimmune diseases and COPD. Exclusion criteria were the presence of the above-mentioned diseases, direct exposure at any time to pesticides and/or herbicides and the lack of will of the participant.

MN test. The MN test is an official regulatory 'tool' in the European Legislation (B.12, Regulation 440/2008/EC) validated by OECD (22). Whole blood (0.5 ml) was added to 6.5 ml Ham's F-10 medium (Gibco/Thermo Fisher Scientific, Waltham, MA USA), 1.5 ml fetal bovine serum (Standard Fetal Bovine Serum, certified, US origin, Gibco/Thermo Fisher Scientific), and 0.3 ml phytohemagglutinin M (PHA-M; 10 ml, Thermo Fisher Scientific). Cultures were incubated at 37˚C for a period of 72 h. Six micrograms per milliliter of cytochalasin-B (white to off-white powder, ≥98% 5 mg; Acros Organics, Inc./Thermo Fisher Scientific) was added 44 h following culture initiation. Peripheral blood lymphocytes (PBLs) were collected by centrifugation at 400 x g (1,500 rpm) at 20˚C for 25 min at 72 h post-incubation. A mild hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v) was added to the cell solution and left for 3 min at room temperature. The cells were fixed with a methanol:acetic acid solution (5:1, v/v) placed on microscope slides and stained with Giemsa (Gibco/Thermo

Table I. Patient characteristics (age, sex, ECOG performance status, location of the primary tumor, number of metastatic sites, BRAFV600E status, KRAS exon 2 status, NRAS status, MMR status).

Characteristic	N=25	%
Median/mean age (range), years	67/66.04 (50-75)	
Sex		
Male	12	48
Female	13	52
Performance status (ECOG)		
0	22	88
1	3	12
Location		
Right-sided	6	24
Left-sided	19	76
Median/mean number of metastatic sites (range)		
Liver	3.5/4.8 (0-20)	
Lung	3/3.4 (0-10)	
Lymph nodes	0/2.33 (0-13)	
Peritoneum	0/1.33 (0-7)	
BRAS^{V600E} status		
WT	13	52
Mutant	2	10
Unknown	10	40
KRAS exon 2 mutation		
WT	13	52
Mutant	10	40
Unknown	2	8
NRAS mutation		
WT	11	44
Mutant	2	8
Unknown	12	48
MMR status		
Proficient	9	36
Deficient	2	8
Unknown	14	56

Fisher Scientific) 15% at 25˚C for 30 min, as previously described (23,24). The slides were then placed under a Nikon Eclipse E200 microscope (Nikon Holdings Europe B.V., Amsterdam, The Netherlands) where the binucleated cells (BN cells) and MN were viewed. One thousand BN cells with an intact cytoplasm were scored per slide for each sample, in order to calculate the MNf. Standard criteria were used for scoring the MN (25). The cytokinesis block proliferation index (CBPI) is given by the following equation:

$$CBPI = \frac{M_1 + 2M_2 + 3(M_3 + M_4)}{N}$$

where M1, M2, M3 and M4 correspond to the number of cells with 1, 2, 3, and 4 nuclei and 'N' is the total number of cells.

Table II. Patient data regarding the therapeutic protocol, biological agent and BMI.

Patient no.	Chemotherapy	Biologic factor	BMI
1	FOLFIRI	No	34.7
2	FOLFOX	Cetuximab	25.76
3	FOLFIRI	Bevacizumab	20.68
4	FOLFIRI	Bevacizumab	29.17
5	FOLFIRI	No	32.46
6	FOLFOX	Cetuximab	32.46
7	FOLFIRI	No	20.44
8	FOLFIRI	Aflibercept	25.24
9	FOLFIRI	Aflibercept	36.48
10	FOLFIRI	Aflibercept	37.63
11	FOLFOX	Aflibercept	41.59
12	FOLFOX	Cetuximab	25.71
13	FOLFIRI	Cetuximab	31.16
14	FOLFOX	Bevacizumab	26.21
15	FOLFOX	No	32.0
16	FOLFIRI	Aflibercept	30.77
17	FOLFIRI	Cetuximab	24.14
18	FOLFIRI	Bevacizumab	21.87
19	FOLFOX	No	24.03
20	FOLFOX	Bevacizumab	25.83
21	FOLFOX	Bevacizumab	32.71
22	FOLFOX	No	26.44
23	FOLFOX	No	25.53
24	FOLFOX	Panitumumab	18.36
25	FOLFOX	Panitumumab	18.75

BMI, body mass index; FOLFIRI, folinic acid with 5-fluorouracil and irinotecan; FOLFOX, folinic acid with 5-fluorouracil and oxaliplatin.

These parameters were calculated by counting 2,000 cells, in order to determine the possible cytotoxic effects, as previously described (26).

Statistical analysis. Statistical analysis of the MN data was performed with the G-test for independence on 2x2 tables. The Chi-squared test was used for the analysis of the CBPI data. The level of significance was set at 0.05. One-way ANOVA was applied to estimate differences between 3 groups. Mean plots with error bars and bar charts were used for the graphical presentation of the data. The IBM SPSS Statistics Package 21.0 was used for data analysis and for the graphic representation of the data. The level of acceptance of null hypotheses was set at the 0.05 level.

Results

The patient characteristics are shown in Table I, while the type of chemotherapy, the biological factor and the BMI of each patient are presented in Table II. Out of the 25 patients, 12 were treated with FOLFIRI and 13 with FOLFOX, while 18 of them were additionally treated with a biological agent

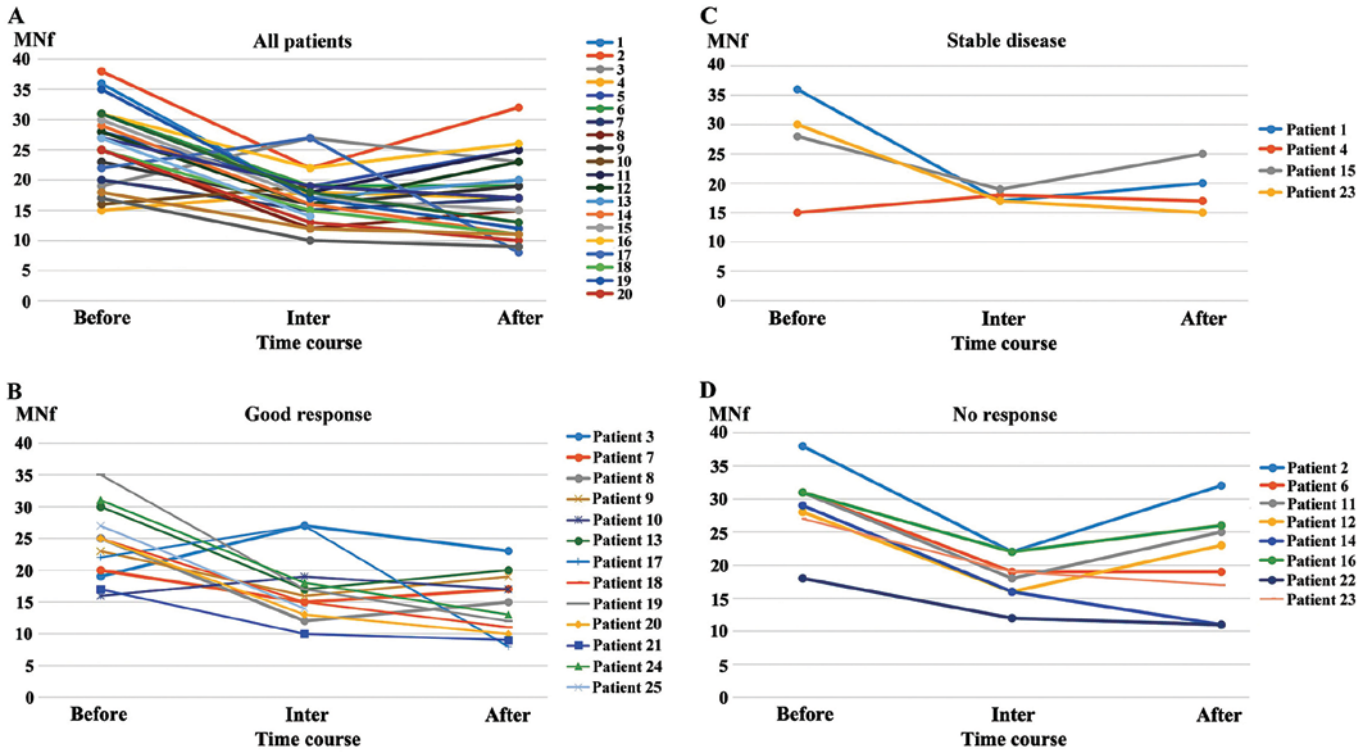

Figure 1. (A) MNf of all patients for the three time-points exhibiting a mixed 'v' and 'Λ' trend. (B) MNf of the good response group presenting a shallow 'v' trend. (C) MNf of the stable disease group presenting a very shallow 'v' trend. (D) MNf of the no response group presenting a deep 'v' trend. MNf, micronuclei frequency.

(cetuximab, aflibercept, bevacizumab or panitumumab). The mean BMI was 28.07 (ranging from 18.36 to 41.59).

In the control group (10 individuals), the mean values of binucleated cells with micronuclei (BNMN), and MN and CBPI values were 6.91±1.14, 7.91±1.14 and 1.34±0.04 respectively. Fig. 1 illustrates the MNf trends of each patient when interpreted as one group (Fig. 1A), as a good response group (Fig. 1B), as a stable disease group (Fig. 1C) and as a no response group (Fig. 1D) across their treatment (at the beginning, middle and end). Fig. 1A exhibits a mixed 'v' and 'Λ' trend, Fig. 1Ba shallow 'v' trend, Fig. 1C a very shallow 'v' trend, and Fig. 1Da deep 'v' trend.

Data regarding the mean values of BNMN, MN and CBPI and the related P-values when patients were treated as a solid group are presented in Table III. Table IIIA shows the data from the comparison of all the patient mean MNf, BNMN and CBPI values to those of the controls. For all time-points (before, middle and after treatment) the patient mean BNMN and MNf values [BNMN: Before, 23.84±5.58 (P<0.001); middle, 15.56±3.54 (P=0.004); and after, 15.21±5.53 (P=0.006); MNf: Before, 26.28±6.30 (P<0.001); middle, 17.40±4.08 (P=0.003); and after, 17.29±6.19 (P=0.004)] were significantly higher compared to those of the controls. However, no significant differences were observed for CBPI (before, 1.30±0.05; middle, 1.32±0.06; and after, 1.31±0.02).

Table IIIB shows the results from the comparison between patients with BMI <25 (7 patients) and BMI ≥25 but ≤30 (8 patients) before therapy. The mean BNMN and MNf values were as follows: (BNMN: BMI <25, 24.00±5.69; BMI ≥25 but ≤30, 23.88±6.79; MNf: BMI <25, 25.57±5.88; BMI ≥25 but ≤30, 26.13±7.26) and did not exhibit any significant differences (BMI 25-30 vs. BMI <25; P=0.98 and P=0.91, respectively). Furthermore, the results from the comparison between patients with BMI <25 and BMI >30 (10 patients) before therapy (BNMN: BMI <25, 24.00±5.69; and BMI >30, 23.70±5.06; MNf: BMI <25, 25.57±5.88; and BMI >30, 26.50±7.97) also did not exhibit any significant difference (P=0.95 and P=0.85, respectively). The mean CBPI was almost the same for all the BMI groups (BMI <25, 1.29±0.05; BMI ≤25 but ≤30, 1.31±0.05; and BMI >30, 1.32±0.06).

Table IIIC shows the results when all the patient mean BNMN, MNf and CBPI values at the middle (BNMN, 15.56±3.54; MNf, 17.40±4.08; CBPI, 1.32±0.06) and at the end (after) (BNMN, 15.21±5.53; MNf, 17.29±6.19; CBPI, 1.31±0.02) were compared to those before treatment (BNMN, 23.84±5.58; MNf, 26.28±6.30; CBPI, 1.30±0.05). The comparison of the mean BNMN, MNf and CBPI values at the middle against those at the beginning of treatment revealed that the mean BNMN values were not significantly lower (P=0.05), while the mean MNf values were (P=0.04). The comparison of the mean BNMN, MNf and CBPI values at the end against those at the beginning of treatment revealed that both the mean BNMN and MNf values were significantly lower (P=0.04 and P=0.04, respectively). The CBPI values were again almost the same for both time-points.

Data regarding the mean values of BNMN and MN and the related P-values when patients were divided into subgroups are presented in Table IV. Table IVA shows the results from the comparison of the samples before treatment from the patients with a good response (13 patients) against those who were stable (4 patients) and those with no response (8 patients). The mean BNMN values before treatment for the good, stable and no response groups were 22.31±5.28, 23.00±6.38 and 26.75±5.20, respectively. The comparison between the groups did not reveal any

Table III. Statistical analysis of MN assay in cultures of peripheral blood lymphocytes showing BN scored, mean frequency of BNMN, mean frequency of MN and CBPI, for the mean BNMN, MNf and CBPI values.

A, all vs. controls

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
Control	10,000	6.91±1.14			7.91±1.14			1.34±0.04
Before	25,000	23.84±5.58	25.47	**<0.001**	26.28±6.30	26.71	**<0.001**	1.30±0.05
Middle	25,000	15.56±3.54	8.03	**0.004**	17.40±4.08	8.54	**0.003**	1.32±0.06
After	25,000	15.21±5.53	7.47	**0.006**	17.29±6.19	8.37	**0.004**	1.31±0.02

B, BMI 25-30 vs. <25 and >30 vs. <25

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
BMI <25	7,000	24.00±5.69			25.57±5.88			1.29±0.05
BMI 25-30	8,000	23.88±6.79	0.006	0.98	26.13±7.26	0.012	0.91	1.31±0.05
BMI >30	10,000	23.70±5.06	0.004	0.95	26.50±7.97	0.03	0.85	1.32±0.06

C, middle vs. before and after vs. before therapy

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value	CBPI (means ± SE)
Before	25,000	23.84±5.58			26.28±6.30			1.30±0.05
Middle	25,000	15.56±3.54	3.85	**0.05**	17.40±4.08	3.99	**0.04**	1.32±0.06
After	25,000	15.21±5.53	4.24	**0.04**	17.29±6.19	4.11	**0.04**	1.31±0.02

Values in bold font indicate statistically significant differences (P<0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored; 25,000 cells in total for each endpoint); BNMN, binucleated cells with micronuclei; CBPI, cytokinesis block proliferation index; BMI, body mass index.

significant differences (good vs. stable, P=0.88; and good vs. no response, P=0.36). The mean MNf values before treatment for the good, stable and no response group were 24.23±5.60, 27.25±8.85 and 29.13±5.59, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.54; and good vs. no response, P=0.33).

Table IVB shows the results from the comparison of the samples at the middle of treatment from the patients with a good response against those which were stable and those with no response. The mean BNMN values for the good, stable and no response groups at the middle of therapy were 15.23±4.53, 15.75±0.96 and 16.00±2.62, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.89; and good vs. no response, P=0.84). The mean MNf before treatment for the good, stable and no response group were 16.92±5.11, 17.75±0.96 and 18.00±3.34, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.84; and good vs. no response, P=0.79).

Table IVC shows the results from the comparison of the samples at the end of the treatment from the patients with a good response against those with a stable response and those with no response. The mean end BNMN values for the good, stable and no response group were 12.67±4.21, 17.00±3.74 and 18.57±7.04, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.24; and good vs. no response, P=0.12). The mean MNf values before treatment for the good, stable and no response group were 14.50±4.76, 19.25±4.34 and 21.00±7.85, respectively. The comparison between the groups did not reveal any significant differences (good vs. stable, P=0.70; and good vs. no response, P=0.11).

Table IVD shows the results from the comparison of the mean BNMN and MNf values from the good response group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were 22.31±5.28, 15.23±4.53 and 12.67±4.21, respectively. The mean MNf values for the same time-points were 24.23±5.60, 16.92±5.11 and 14.50±4.76, respectively. The comparison between time-points revealed a significant decrease only when after treatment was compared with before treatment, with an insignificant decrease at the middle (BNMN: Before vs. middle, P=0.09; and before vs. after, P=0.01; MNf: Before vs. middle, P=0.09; and before vs. after, P=0.02).

Table IV. Statistical analysis of the mean BNMN and MNf at different time-points.

A, before therapy: Stable vs. good and no response (No res vs. good).

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	22.31±5.28			24.23±5.60		
Stable	4,000	23.00±6.38	0.02	0.88	27.25±8.85	0.37	0.54
No res	8,000	26.75±5.20	0.85	0.36	29.13±5.59	0.95	0.33

B, at the middle of treatment: Stable vs. good and No res vs. good.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	15.23±4.53			16.92±5.11		
Stable	4,000	15.75±0.96	0.02	0.89	17.75±0.96	0.04	0.84
No res	8,000	16.00±2.62	0.04	0.84	18.00±3.34	0.07	0.79

C, after therapy: Stable vs. good and No res vs. good.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Good	13,000	12.67±4.21			14.50±4.76		
Stable	4,000	17.00±3.74	1.35	0.24	19.25±4.34	0.15	0.70
No res	8,000	18.57±7.04	2.43	0.12	21.00±7.85	2.60	0.11

D, good response: Middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	13,000	22.31±5.28			24.23±5.60		
Middle	13,000	15.23±4.53	2.93	0.09	16.92±5.11	2.84	0.09
After	13,000	12.67±4.21	6.06	**0.01**	14.50±4.76	5.52	**0.02**

E, stable response: middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	4,000	23.00±6.38			27.25±8.85		
Middle	4,000	15.75±0.96	2.97	0.08	17.75±0.96	4.45	**0.03**
After	4,000	17.00±3.74	1.94	0.16	19.25±4.34	3.01	0.08

F, no response: middle vs. before and after vs. before.

Group	BN cells scored	BNMN (means ± SE)	G	P-value	MNf (means ± SE)	G	P-value
Before	8,000	26.75±5.20			29.13±5.59		
Middle	8,000	16.00±2.62	6.11	**0.01**	18.00±3.34	5.91	**0.02**
After	8,000	18.57±7.04	3.24	0.07	21.00±7.85	2.87	0.09

Values in bold font indicate statistically significant differences (P<0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored; 25,000 cells in total for each endpoint); BNMN, binucleated cells with micronuclei.

Table IVE shows the results from the comparison of the mean BNMN and MNf values from the stable group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were

23.00±6.38, 15.75±0.96 and 17.00±3.74, respectively. The mean MNf values for the same time-points were 27.25±8.85, 17.75±0.96 and 19.25±4.34, respectively. The comparison between time-points revealed a significant decrease only for MNf when middle was compared with before treatment, while BNMN for the same time-point exhibited an insignificant decrease. At the end of the therapy, both the BNMN and MNf values increased so that there were no significant difference between before and after therapy (BNMN: Before vs. middle, P=0.08; and before vs. after, P=0.16; MNf: Before vs. middle, P=0.03; and before vs. after, P=0.08).

Table IVF shows the results from the comparison of mean BNMN and MNf values from the no response group before therapy against those at the middle and after therapy. The mean BNMN values before, middle and after therapy were 26.75±5.20, 16.00±2.62 and 18.57±7.04, respectively. The mean MNf values for the same time-points were 29.13±5.59, 18.00±3.34 and 21.00±7.85, respectively. The comparison between time-points revealed a significant decrease only when middle was compared with before therapy both for BNMN and MNf. On the contrary, at the end of the therapy, both BNMN and MNf increased so that there was no significant difference between before and after treatment (BNMN: before vs. middle, P=0.01; and before vs. after, 0.07; MNf: before vs. middle, P=0.02; and before vs. after, P=0.09).

Discussion

The results of the current study indicated that patients diagnosed with metastatic CRC, regardless of sex and BMI, had high rates of BNMN and MNf. This was found both before and throughout the systemic therapy, even though they tended to decrease after therapy, but never to the degree of the individuals without cancer. In parallel, they had the same CBPI with healthy individuals that remained stable throughout treatment, while no change in the CBPI was evidenced at any point time or for any group.

It is well established that MN assay is a sensitive indicator of genomic damages of exogenous and endogenous origin (23,27). MNf in PBLs represents an indirect, intracellular indicator of chromosomal and genomic instability (high levels of MN are indicative of extended damages of the DNA repair system and in chromosomal division) (17,28-30). It has been proven that, even though MNf does not differ between the two sexes (31), it does between young and older and between normal-weight and obese individuals as a result of the accumulation of genetic damage (31,32). These facts support the hypothesis that the CBMN assay can be used as an indicator of the genotoxic and cytotoxic state (33). Indeed, it has previously been concluded that high levels of MN are linked to cancer (16). Moreover, a number of theories support the hypothesis that MNf can be used as a tool for cancer prognosis (18,19,34); however, they all agree that further investigations are required to verify this claim.

This study focused on the evaluation of MNf as a potential prognostic/predictive biomarker for CRC monitoring in a rather common group of patients with CRC, those with distant metastases (stage IV disease). For this purpose, 25 patients with stage IV CRC from a single oncologic center were included. Based on the current therapeutic guidelines for stage IV CRC, these patients underwent treatment with either FOLFOX or FOLFIRI with an addition of a biological factor based on their underlying genetic status (RAS and BRAF mutations). Folinic acid and 5-fluorouracil are the common compounds of the FOLFOX and FOLFIRI regimens, while oxaliplatin and irinotecan are the compounds that differentiate them, respectively. Bibliographic data have indicated that FA is an anti-clastogenic agent which significantly reduces the percentage of BNMN (35). It has been found that oxaliplatin induces cytogenetic damage (BNMN) through its clastogenic action, possibly through interfering with topoisomerase II (36). As regards irinotecan, Kopjar *et al*, using the CBMN assay, observed a dose-dependent increase in MNf in an *in vitro* study with human lymphocytes (37). Another study on irinotecan also found a significant increase of BNMN, but in a non-dose-dependent manner (38). However, to the best of our knowledge, there is no study available to date estimating the MNf and BNMN using the actual combination of FOLFOX or FOLFIRI with or without biological agents. Moreover, to the best of our knowledge, this study is the first one conducted with such a patient group and, thus, any interpretation of the data presented will be based mainly on data coming from different patient groups and thus should be treated accordingly.

CBPI is a tool widely used not only to better understand the BNMN results, but also to estimate any cytotoxic effect from chemical agents on cell cultures that use cytochalasin B expressed by an altered proliferation cells (38). As regards the best understanding of BNMN results, when CBPI is indifferent between time-points, then MNf results are comparable and any fluctuation of MNf can be attributed solely to the disease and/or the systemic treatment. As for the cytotoxicity, when the CBPI value is close to one, there is no cytotoxic event. However, in order to extract safer conclusions regarding cytotoxicity, patient CBPIs are compared to those of the control and not to the unit. In this study, if we address all patients as one solid group, before the beginning of the therapy, we can see that there was no difference in their CBPI values compared with the healthy individuals, suggesting no cytotoxicity from the disease. Moreover, we can see that CBPI remained almost the same throughout the duration of therapy. Thus, the combination of the disease and chemotherapy again did not lead to cytotoxicity. Therefore, it is safe to say that the MNf results are indicative of the patients' condition. Since sex does not affect MNf and the age group of our patients was the same (between 50 and 75 years old), the main parameters that had to be examined as to whether they affect MNf were BMI and malignancy per se. For the former case, patients were divided based on their BMI into 3 groups (BMI <25, BMI ≤25 but ≤30 and BMI >30). Statistical analysis of the MNf before the beginning of the treatment revealed no statistical differences (Table IIIB). For this reason, BMI was excluded from the final interpretation. The comparison of the MNf and BNMN scores between the patients before the systemic treatment and the healthy individuals (matched for sex, age and BMI) revealed significantly higher rates for both indexes (P<0.001). In fact, this significantly higher rate of MNf and BNMN was maintained throughout treatment (Table IIIA). Thus, it is reasonable to assume that the increased rates of MNf and BNMN are due to cancer. A following comparison of the mean BNMN and MNf of all patients revealed that BNMN decreased insignificantly at the middle and significantly at

the end (P<0.05), while MNf was significantly lower for both time-points (P=0.04 and P=0.04 respectively) (Table IIIC).

Based on the RECIST 1.1 criteria, we further divided the patients into the 'good response', 'stable disease' and 'no response' groups. The subsequent analysis revealed some very interesting data. First of all, when each subgroup was compared to the other for the same time-point, no significant differences were revealed both for the mean BNMN and MNf values. However, the subsequent comparison between time-points of the same group revealed that the 'good response' group had a declining trend for BNMN and MNf with an insignificant decrease at the middle (P=0.09 for both), and a significant one at the end of the therapy (P=0.01 and P=0.02, respectively) exhibiting a 'shallow v trend' (Fig. 1A). The same analysis was performed for the 'stable disease' group revealing a significant) decrease followed by an increase, making the MNf difference between before and after treatment insignificant (Fig. 1C). The 'no response' group exhibited a significant decrease at the middle both for BNMN and MNf (P=0.01 and P=0.02, respectively). Interestingly though, the trend was reversed at the end of the therapy, where both the BNMN and MNf values increased to such an extent, that no significant difference was evident anymore, exhibiting a 'deep v trend' (Fig. 1D).

Overall, there is a clear genotoxic state in the PBLs of patients represented by the very high mean MNf before therapy. This genotoxic state depicts the great cancer load at that time. After the first trimester of the therapy, the decrease in the mean MNf reflects the response of the organism to the treatment accomplished by the depletion of the sensitive cancer clone. However, the following increase of mean MNf (but never to the degree before treatment) raises a challenge for its clinical interpretation. The first scenario is that of a 'gradual emergence of a resistant clone'. First, sensitive cancer cells are depleted and so MNf and BNMN decrease. Subsequently, resistant ones emerge as they do not have to compete for energy or oxygen supply. In fact, this scenario could explain the fluctuations in MNf observed in the 3 response groups during the therapy. At the middle of therapy, both the good and no response groups began killing sensitive cells and decreased their MNf numbers, while the stable group did not. While the good responders then continued to deplete sensitive cancer cells, the non-responders began to increase cancer cells and their MNf increases accordingly, while the stable group maintained almost the same cell number and MNf. The second scenario is the 'long-term chemo-effect'. As mentioned before, both oxaliplatin and irinotecan increase MNf. Thus, while the cancer load decreases and the drug accumulation is not yet at its peak, the MNf also decreases. However, as the rate of cancer cells decrease diminishes and the accumulation of the drug reaches its zenith, the MN-increasing properties of oxaliplatin and irinotecan become evident. This scenario can also explain why at the end of the treatment the response group did not differ significantly, in terms of mean BNMN and MNf, than the other 2 groups, even though their cancer burden was reduced by >30%. In other words, systemic treatment increased MNf and prevented a cell number difference to be seen. Interestingly though, even if the majority of the patients exhibited the 'v'-shaped trend of MNf, there were 4 patients who exhibited a reverse 'v'-shaped trend, with an increase of mean MNf at 3 months, and a subsequent decrease at 6 months, as shown in Fig. 1. It is noteworthy that these 4 patients who did not follow the 'v'-shaped trend as the rest of the participants, but rather an inverted 'v', were proven to share the same therapy with a combination therapy of FOLFIRI and some type of biological agent. Whichever the case may be, as exhibited by the results from the good and the poor response groups, MNf was not associated with tumor response.

The key is to identify the exact time when the relapse or stability of the disease occurs and is first depicted in MNf by a certain increase of it. In doing so, we would be able to achieve a better tailoring of the therapy and at the same time we will be a step closer towards personalized treatment with a possible shortening of the chemotherapy duration. This in turn would positively affect not only patients, in terms of less side-effects as a consequence of tailored systemic therapy, but also the health care system due to the decreased financial burden of shortened systemic therapy. However, more patients and even more sampling points would be required in order to successfully identify the true nadir of MNf.

The findings of this study reveal an association, firstly between MNf and CRC per se, with significantly elevated MN rates at all time-points and, secondly between MNf and response to treatment, where a good response was evidenced by the significantly low rates at the end of treatment and a bad response by the maintenance of high rates at the end. Despite the fact that the results of the current study are in the same line of evidence with previously published data (15-19), they should be interpreted with caution and would be used as hypothesis-generated. We aim to continue this research in a prospective larger group of patients with metastatic CRC in order to validate the findings of the current study and establish the prognostic and predictive significance of MNf in this setting.

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Availability of data and materials

All data generated or analyzed during this study is included in this published article or are available from the corresponding author upon reasonable request.

Authors' contributions

TKN conducted the experiments, interpreted the data and wrote the manuscript. PDS performed the analysis and interpreted the data. PA conducted the experiments and wrote the manuscript. KK conducted the experiments and wrote the manuscript.

TMS drafted, interpreted the data and critically revised the article. DAS conceived and designed, critically reviewed and supervised the article. AT conceived and designed, critically revised, provided laboratory infrastructure and was responsible for the critical revision of the article for important intellectual content. JS provided blood samples, edited the manuscript and was responsible for the critical revision of the article for important intellectual content. JT conceived and designed the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Blood and information of patients were obtained with written informed consent. Procedures involving patients in this study were approved by the Human Ethics Committee at the University Hospital of Heraklion on December 2016.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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Novel Prognostic Biomarkers in Metastatic and Locally Advanced Colorectal Cancer: Micronuclei Frequency and Telomerase Activity in Peripheral Blood Lymphocytes

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Purpose: Due to the current practice on colorectal cancer (CRC) management, chemoresistance is most often recognized at the end of the treatment. Therefore, effective and easy-to-use prognostic biomarkers are needed.

Experimental Design: We evaluated the prognostic significance of two novel CRC biomarkers: a) micronuclei frequency (MNf) in 55 metastatic CRC (mCRC) and 21 locally advanced rectal cancer (laRC) patients using cytokinesis block micronucleus assay (CBMN assay) and b) telomerase activity (TA) in 23 mCRC and five laRC patients using TRAP-ELISA. Both biomarkers were evaluated in peripheral blood lymphocytes (PBLs) before, at the middle, and at the end of the therapy (approximately 0, 3, and 6 months) for mCRC patients before, at the end of the therapy, and after surgery for laRC patients.

Results: Overall, MNf demonstrated significant prognostic value since a decrease of MNf less than 29% between middle and initial MNf measurements can discriminate between progressive and stable/responsive disease with sensitivity of 36% and specificity of 87.0% while being able to identify responsive disease with sensitivity of 72.7% and specificity of 59.3%. On the other hand, TA presented a significant trend of increase ($p = 0.07$) in patients with progressive disease at the middle measurement.

Conclusions: The findings of this study suggest that the MN frequency may serve as a promising prognostic biomarker for the monitoring of the treatment response of patients with CRC, while TA should be evaluated in a larger group of patients to further validate its significance.

Keywords: metastatic colorectal cancer, locally advanced rectal cancer, micronuclei frequency, telomerase activity, biomarkers, prognosis

INTRODUCTION

Colorectal cancer is considered as one of the leading causes of cancer-related morbidity and mortality worldwide for both sexes. It was estimated that in 2020 147,950 individuals would be diagnosed with CRC in the United States (70.7% would suffer from colon and 29.3% from rectal cancer) while 53,200 patients would die from the disease (1). Projection in 2021 has not improved since it is estimated that 149,500 individuals will be affected and 52,980 will die by it (2). CRC patients with distant metastases present the worst prognosis since a significant number of them develop resistance to their therapy. Unfortunately, diagnosis of chemoresistance is most often delayed, allowing for cancer progression to take place before these patients receive second or third line treatments. At the same time, healthcare systems are dealing with an immense financial burden as a result of these treatments. Therefore, it is crucial to identify accurate, cost efficient, and easy-to-use tools that will provide valuable prognostic and predictive information. A great body of evidence indicates molecular biomarkers as promising candidates for this purpose (3). In our previous work on MNf in mCRC (4), we demonstrated that in accordance to already published data (5), mCRC results in higher MNf than that of healthy individuals. Moreover, chemotherapy was found to have a direct effect on MNf as documented in the middle of the therapy. In addition, patients with complete or partial response had further decrease in MNf at the end of the treatment in contrast to those with stable or progressive disease. In fact, this finding suggests that a decrease of cancer load can be identified by the concomitant decrease of MNf, while an increase of the cancer load (due to the emergence of a chemoresistant cluster of cancer-cells) will result in an increase of MNf, but not to the same level as before treatment. Therefore, our team focused on validating the clinical and possible prognostic value of two novel biomarkers (MNf and TA) for laCRC and mCRC. These biomarkers were chosen on the basis of their close relation to chromosomal instability (CIN) and aberrant genetic function, both major hallmarks in colorectal carcinogenesis (6).

Micronuclei (MNs), also known as Howell–Jolly bodies, are particles formed during anaphase, since part of the genetic material fails to follow the rest of the chromosomes and therefore is not included in the daughter nucleus during telophase. This results in a smaller "nucleus" close to the main one. It is exhibited that MN formation can be attributed primarily to mitotic spindle failure, kinetochore damage, centromeric DNA hypomethylation, and defective control in the cell cycle system (7). Nonetheless, their presence in a healthy individual is not unusual although they tend to be more common among people adhering to unhealthy lifestyles (8). Generally, males tend to have lower MNf than females, and younger individuals have lower MNf than the older individuals (9). MNf estimation is well-established as an indicator of genetic damage of any cause, especially as an exposure biomonitoring against carcinogens (10, 11). Numerous studies have shown that MNf is a sensitive biomarker of various types of cancers such as lung, bladder, and CRC, suggesting that cancer patients exhibit higher MNf than healthy individuals (4, 5). Although a number of studies have explored MNf in CRC (12, 13), it is not clear what is the course of MNf in the long-term and especially how MNf is correlated with prognosis.

Telomerase, the regulating enzyme of telomeres' length, is an enzymatic complex consisting of two subunits, the catalytic subunit, the human telomerase reverse transcriptase (hTERT), and a template, the telomerase RNA component (TERC). Notwithstanding that, the telomeres' length is induced in each cell cycle in a lower rate, endorsing cellular senescence (14). In non-cancerous somatic cells, TA is undetectable or present at low levels. Cellular senescence is a key barrier against cancer, which implies that cancer cells have been transformed to immortal cells. This fact requires increased levels of TA, in order not to decrease telomere's length. This mechanism is explained by the hTERT promoter, whose upregulated expression is promoted by differential hTERT gene expression in neoplastic and normal cells. For example, Chen et al. demonstrated that a net increase of hTERT units is possible through upregulation of SPT5, a tumor-specific hTERT promoter-binding protein encoded by the upregulated SUPT5H gene (15), while Ling Zhang et al. using the HCT-116 cell line (a KRAS mutated line), exhibited increased TA *via* upregulation of the T-STAR gene (which encodes a number of proteins responsible for multiple functions in pre-mRNA splicing, signaling, and cell cycle control) (16). Given the implication of telomeres' length in CRC, TA has attracted scientific interest as well. Jian Zou et al. identified that telomerase is found to be activated in 90% of malignant tumors (17). Interestingly, TA has been detected in early stages of CRC which would mean that it is a determining factor during carcinogenesis (18), while increased hTERT expression and elevated plasma concentration of circulating TERT mRNA have also been identified as an unfavorable independent prognostic marker of overall survival in patients with stage II CRC (19).

MATERIALS AND METHODS

Patients and Study Protocol

From December 2016 to February 2021, 94 consecutive patients treated at the Department of Medical Oncology, University Hospital of Heraklion were evaluated for participation in this study. Inclusion criteria were: I) Patients with radiologic evidence of mCRC documented by computed tomography (CT) and/or magnetic resonance imaging (MRI) presenting measurable disease treated with first line systemic treatment according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (20), II) Patients with radiologic evidence of laRC, documented by abdominal and chest CT and abdominal and pelvic MRI presenting measurable disease and receiving induction chemotherapy according to the Hellenic Society of Medical Oncologists (HeSMO) guidelines (21). Exclusion criteria were as follows: I) Failure to complete the therapeutic regimen for any reason (toxicity, refusal of the patient, or death), II) Refusal of the patient to attend the study III) Synchronous second primary cancer at the time of enrollment and/or therapy. Out of 94 patients evaluated, 85 were found eligible and were included; however, only 76 managed to complete the study (55 mCRC and 21 laRC) due to the fact that five patients

presented increased toxicity and had to stop while another four died prior to completion of the study (**Figure 1**). We decided to use the embryologic origin of each part of the colon in order to divide primary location of the lesion on the right and left sides (caecum, ascending colon, and proximal 2/3 of the transverse colon from the midgut and therefore right colon, distal third of the transverse, the descending, sigmoid colon, and the rectum from the hindgut and therefore left colon). The protocol for this study has been approved by the Ethics Committee for Patients and Biological Material of the University Hospital of Heraklion (Heraklion, Greece). All participants signed an informed consent agreement. All samples generated by this study were anonymized, and personal data was managed according to the EU General Data Protection Regulation (GDPR).

Therapy Selection for mCRC

Based on the chemotherapeutic protocol that was selected, mCRC patients received one of the following therapies: I) folinic acid with 5-fluorouracil and oxaliplatin (FOLFOX), II) folinic acid with 5-fluorouracil and irinotecan (FOLFIRI), or III) folinic acid with 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI). Based on the genetic profile of each patient, a biological factor could be used.

Therapy Selection for laRC

Patients with laRC were informed about their therapeutic strategy: Induction chemotherapy and then operation for RC. Chemotherapeutic protocols were FOLFOX and capecitabine and oxaliplatin (CAPOX).

Response Evaluation

The RECIST criteria version 1.1 were used as the gold standard for the evaluation of the treatment response (22). Using these criteria, patients were evaluated at the end of the therapy and were divided into four subgroups: complete response (disappearance of all target lesions), partial response (at least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters), stable disease (neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for partial disease, taking as reference the smallest sum diameters) and progression (at least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study or the appearance of one or more new lesions).

Blood Sampling

Peripheral blood samples were collected at predetermined time-points. For mCRC patients, these were before the beginning of the treatment, in the middle of it, and at the end of treatment (approximately 0, 3, and 6 months of treatment, respectively). For laRC patients, samples were taken before the beginning of chemotherapy and at the end. We also took blood samples two weeks postoperatively from 12 laRC patients. All blood samples were stored in 5°C until processing within 48 h from sampling.

Control Group

The control group was constituted by 25 healthy individuals with no medical condition after having normal colonoscopy. All individuals provided an informed written consent. Inclusion

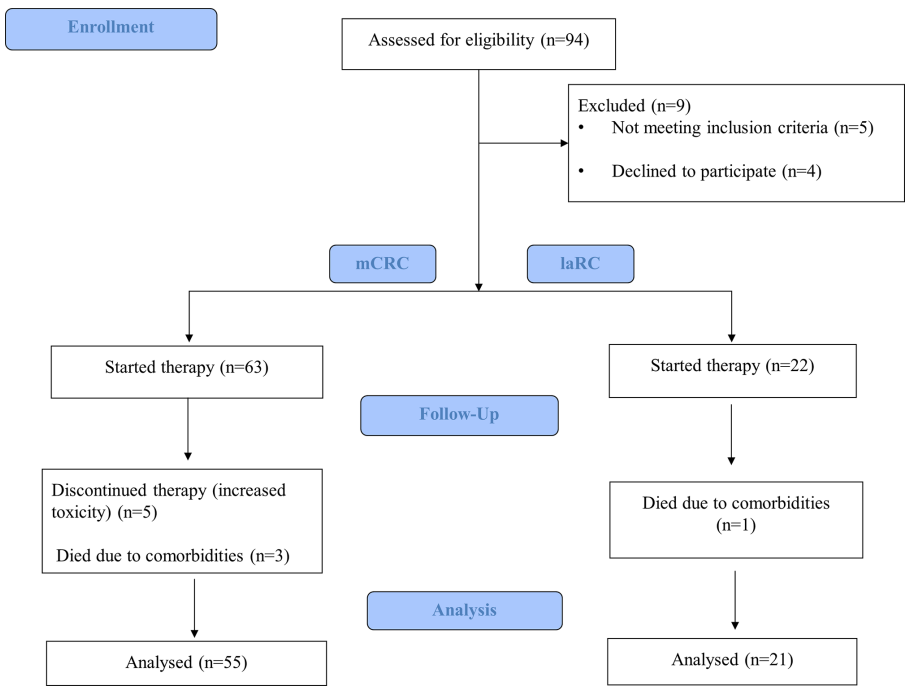

FIGURE 1 | CONSORT flow diagram of our study.

criteria were: age between 45 and 75 years old, medical history free of cancer, autoimmune diseases, diabetes mellitus type I or II, and chronic obstructive pulmonary disease (COPD), non-smokers or no smoking habits for the last ten years, and no consumption of immune-modifying medication. Exclusion criteria were the presence of the above-mentioned diseases, direct exposure at any time (domestic or occupational) to pesticides, herbicides, organic solvents, or any persistent organic pollutant; for women, additional exclusion criteria were the use of oral contraceptives and the will not to participate in the study.

MN Test

Laboratory preparation for MNf quantification follows the description we have presented earlier (4). Standard criteria were used for scoring the BNMN: 1) the cells should be binucleated, 2) the two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary, 3) the two nuclei in a binucleated cell should be approximately equal in size, staining pattern, and staining intensity, 4) the two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter, 5) the two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable, 6) the cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells. One thousand binucleated (BN) cells with an intact cytoplasm were scored per slide for each sample in order to calculate the number of binucleated cells with micronuclei (BNMN) and thereafter MNf. Criteria for scoring MN were: 1) the diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively, 2) MNs are round or oval in shape, 3) MNs are non-refractile, and they can therefore be readily distinguished from artefacts such as staining particles, 4) MNs are not linked or connected to the main nuclei, 5) MNs may touch but not overlap the main nuclei, and the micronuclear boundary should be distinguishable from the nuclear boundary, 6) MNs usually have the same staining intensity as the main nuclei but occasionally staining may be more intense (23, 24). The cytokinesis block proliferation index (CBPI) is given by the following equation:

$$CBPI = \frac{M_1 + 2M_2 + 3(M_3 + M_4)}{N}$$

where M1, M2, M3, and M4 correspond to the number of cells with one, two, three, and four nuclei, respectively, and 'N' is the total number of cells. For CBPI calculation, 2,000 cells were counted. CBPI is a tool that is used in order to better understand BNMN results from cell cultures where cytochalasin B is used. Moreover, it is able to provide substantial information regarding possible cytotoxic effects (necrosis, apoptosis, or cytostasis) on the cell culture induced by any chemical agents. If CBPI remains close to the numeric value of one, then there is no cytotoxic event. Moreover, should it remain almost the same between time-points, then MNf results are comparable, and any fluctuation of MNf can be attributed solely to the parameter of interest (in our case CRC and/or the systemic treatment). These parameters were calculated, in order to determine the possible cytotoxic effects, as previously described (25–27).

Telomerase Activity Estimation: Polymerase Chain Reaction–Enzyme Linked Immune Sorbent Assay

TA was measured in 28 patients, whose characteristics are presented in **Table 1** at three time points: before (at the beginning of the therapy), middle (at the middle of therapy for mCRC, and at the end of therapy for laRC) and after (at the end of therapy for mCRC and after surgery for laRC). Telomerase activity was evaluated by photometric enzyme immunoassay for the detection of telomerase activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP). Peripheral blood mononuclear cells (PBMCs) were harvested from the blood samples by Ficoll–Hypaque gradient centrifugation as described by Tsirpanlis et al. (28), and TRAP-ELISA was conducted using TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA) following the manufacturer's manual (29) and Kara et al. (30). In order to achieve higher data reliability, all samples were tested in triplicates. TA was expressed as a totality and as per outcome (progression, stable, partial and complete response).

Statistical Analysis

Frequency data were analyzed using non-parametric statistics. Pearson's Chi-square test (χ^2) was applied to estimate differences in proportions of patients' and disease characteristics (**Table 1**). In order to examine TA differences and percentage differences of MNf (%DMNf) between two groups (*e.g.* mCRC *vs* IaRC), the Mann–Witney test was applied. Whereas, in order to examine TA differences and %DMNf for more than two groups (*e.g.* disease response) Kruskal–Wallis comparisons were applied. Comparison of counts of MNf was assessed using G-test when bivariate comparisons of before, middle, and after therapy sampling points were made. The Chi-squared test was used for the analysis of the CBPI values., Due to the small number of cases, a crude discrimination limit between responses was established using %DMNf as an indicator. The %DMNf definition between middle and before was set by the formula

$$\%\,DMNf = \frac{MNf_{middle} - MNf_{before}}{MNf_{before}} 100\,\%$$

ROC curve analysis, corresponding diagrams of sensitivity *vs* 1-specificity were applied between %DMNf in a binary response (progressive *vs* stable/partial/complete response and progressive/stable *vs* partial/complete response) according to %DMNf.

IBM SPSS Statistics 26.0 and OpenEpi 3.01 open source epidemiological program (https://www.openepi.com/Menu/OE_Menu.htm) were used for statistical analysis of data and sensitivity analysis. A level of 0.05 was set as level of acceptance.

RESULTS

As presented in **Table 1**, we prospectively studied 76 CRC patients, 55 diagnosed with mCRC and 21 with laRC. In our mCRC group, 29 patients were treated with FOLFOX, 22 with FOLFIRI, and four with FOLFOXIRI (52.7, 40, and 7.3% respectively), while 40 patients received an additional treatment with a biological agent (cetuximab, aflibercept, bevacizumab or panitumumab) based on their genetic profile; 19 were treated with bevacizumab, five with aflibercept, nine with cetuximab, and seven with panitumumab (34.5, 9, 16.3, and 12.7% accordingly). On the other hand, in our laRC group, 12 were treated with FOLFOX (57%) and nine with CAPOX (43%). Based on RECIST criteria for disease response evaluation, our data suggest that the mCRC group had the following results: 13 patients exhibited progressive disease, 18 had stable disease, 23 had partial response, while one had complete response (23.6, 32.7, 41.8, and 1.8% respectively). Regarding the laRC group, two patients had progressive disease, five were stable, 11 presented partial response, and three had complete response (9.5, 23.8, 52.4,

TABLE 1 | Patient characteristics for MNf and TA groups are presented [sex, age, ECOG performance status, chemotherapy, biologic agent, disease response based on the RECIST criteria, KRAS status, NRAS status, BRAF status, mismatch repair (MMR) status, location of the primary lesion, number of metastatic sites].

		MNf		p	TA		P
		mCRC	laRC		mCRC	laRC	
Total Number of patient		55	21		23	5	
Sex, n (%)	**Males**	28 (50.9)	16 (76.2)	0.068	9 (39.0)	3 (60.0)	0.393
	Females	27 (49.1)	5 (23.8)		14 (61.0)	2 (40.0)	
Age, n (%)	**≤40**	0 (0.0)	1 (4.8)	0.435	1 (4.8)	1 (20.0)	0.173
	41-55	13 (23.6)	5 (23.8)		5 (23.8)	3 (60.0)	
	56-70	24 (43.6)	8 (38.1)		8 (38.1)	1 (20.0)	
	70+	18 (32.7)	7 (33.3)		7 (33.3)	0 (0.0)	
ECOG performance status, n (%)	**0**	22 (95.6)	47 (85.5)	0.199	19 (86.4)	5 (100.0)	0.08
	1	1 (4.3)	8 (14.5)		3 (13.6)	0 (0.0)	
Chemotherapy, n(%)	**FOLFOX**	29 (52.7)	12 (57.0)	0.98*	10 (43.5)	4 (80.0)	0.14*
	FOLFIRI	22 (40.0)	0 (0.0)		13 (36.3)	0 (0.0)	
	FOLFOXIRI	4 (7.3)	0 (0.0)		0 (0.0)	0 (0.0)	
	CAPOX	0 (0.0)	9 (43.0)		0 (0.0)	1 (20.0)	
Biological agent, n (%)	**Bevacizumab**	19 (34.5)		NA	7 (30.0)		NA
	Aflibercept	5 (9.0)			5 (22.0)		
	Cetuximab	9 (16.5)			6 (26.0)		
	Panitumumab	7 (12.7)			0 (0.0)		
	No agent	15 (27.2)			5 (22.0)		
Disease response, n (%)	**Progression**	13 (23.6)	2 (9.8)	0.08	6 (20.0)	0 (0.0)	0.25
	Stable disease	18 (32.7)	5 (23.8)		7 (30.0)	3 (60.0)	
	Partial response	23 (41.8)	11 (52.4)		9 (40.0)	1 (20.0)	
	Complete response	1 (1.8)	3 (14.3)		1 (4.0)	1 (20.0)	
KRAS, n (%)	**WT**	25 (45.5)	9 (39.1)	0.61**	Unknown		
	exon 2 mut	18 (32.7)	6 (26.1)		***		
	exon 3 mut	0 (0.0)	0 (0.0)				
	exon 4 mut	1 (1.8)	1 (4.3)				
	Unknown	11 (20.0)	7 (30.4)				
NRAS, n (%)	**WT**	42 (76.4)	15 (65.2)	0.31**	Unknown		
	Mutation	2 (3.6)	2 (8.7)				
	Unknown	11 (20.0)	6 (26.1)				
BRAF, n (%)	**WT**	41 (74.5)	15 (65.2)	0.48**	Unknown		
	V600E mut	4 (7.3)	1 (4.3)				
	Unknown	11 (20.0)	7 (30.4)				
Mismatch repair status, n (%)	**Proficient**	12 (58.2)	21 (91.3)	0.01	Unknown		
	Deficient	2 (3.6)	1 (4.3)				
	Unknown	21 (38.2)	1 (4.2)				
Location of primary lesion, n (%)	**Left**	42 (76.4)	19 (83.0)	0.54	21 (100)	5 (100)	NA
	Right	13 (23.6)	4 (17.0)		0 (0.0)	0 (0.0)	
Metastatic sites [median/mean (range)]	**Liver**	3.6/4.4 (0–20)		NA			NA***
	Lung	3.2/3.5 (0–11)					
	Lymph nodes	0/3.2 (0–14)			0/3.2 (0–6)		
	Peritoneum	0/3.6 (0–8)					

*p-values from comparison of FOLFOX with the rest of chemotherapies, *p-values from comparison between wild-type and mutations,***Unknown: There were no data , ****NA, not applicable.*

and 14.3% respectively). Finally, 32 of the mCRC patients had left-sided primary lesion (76.4%) while 13 had right sided primary lesion (23.63%). We further conducted comparative analysis between mCRC and laRC groups for those characteristics where no data were missing. All comparisons showed that there is an adjustment in demographics and patient's data with the exception of mismatch repair status in MNf dataset (p=0.01).

MN Frequency Evaluation

MNf was measured for all patients in our study, and their characteristics are presented in **Table 1**. Our data from the MN assay on control group, mCRC, and laRC patients are presented in **Tables 2–4** respectively. Based on the absence of a significant difference of the CBPI values between: A) CRC groups and control and B) each sampling point for each response group, we are able to assume that our data is the result of the cancer itself and the different treatments. Our data indicates that MNf is significantly higher in patients with mCRC or laRC than in healthy individuals (G = 41.1, p < 0.0001 and G = 33.76, p < 0.0001 respectively). Moreover, there was no significant difference between mCRC and laRC groups, especially regarding before and middle sampling points (**Figure 2**). MNf analysis for the whole mCRC group revealed that a borderline significance is extracted when the middle of the treatment (middle) is compared to the beginning of the treatment (ZFORE) (p = 0.05), while MNf did not exhibit a further significant decrease at the end point (after) (**Table 3**). After stratification of patients according to their disease response, a relative pattern of a steady drop of MNf was observed. In detail, even though patients with progressive and stable disease exhibit an insignificant decrease of their MNf in the middle (G = 1.60, p = 0.200 and G = 3.48, p = 0.060 respectively) and at the end of their treatment (G = 2.13, p = 0.14 and G = 3.55, p = 0.06 respectively), those with disease progression had lower decrease of MNf than those with stable disease (**Table 3**). On the contrary, patients with partial response presented a statistically significant decrease of their MNf both at the middle and at the end of the treatment (G = 5.16, p = 0.02 and G = 3.94, p = 0.04 respectively) (**Table 3**). However, since there was only one patient with complete response, no statistical analysis of his data was done even though his MNf grossly followed the decreasing trend of those with partial response (before the treatment MNf was 28; at the middle, 18; and at the end, 15). Regarding the laRC group, since our primary objective was to evaluate our biomarkers' prognostic value of therapy response, we tested for MNf after laRC surgery in approximately half of our patients (12 out of 21). Therefore, a difference in counted BN cells is observed for the sampling point "surgery" relative to the other two sampling points (**Table 4**). However, our data indicates a similar decreasing trend as observed in the mCRC group. In detail, a significant decrease of MNf (G = 4.01, p = 0.04) is found at the end of the treatment (sampling point "after") in relation to the beginning (**Table 4**). However, even though patients maintain lower MNf than what they had before treatment, a slight increase is observed after surgery (G = 3.00, p = 0.08) (**Table 4**). When divided in subgroups, our data indicates a steady decrease of MNf that positively correlates to the disease response. Patients with progressive disease present a lower decrease of MNf after treatment (p = 0.24) than those patients with stable disease (G = 2.82, p = 0.09), partial response (G = 4.50, p = 0.03) or complete response (G = 4.77 p = 0.02) (**Table 4**).

Evaluation of MNf as a Prognostic Biomarker

The prognostic significance of MNf in mCRC and laRC patients was roughly established using ROC curve analysis. Variation of MNf expressed as %DMNf between initial and middle measurements was estimated by setting binary outcome variables for two scenarios: Progressive disease *vs* stable/partial/complete response (scenario 1) and progressive/stable disease *vs* partial/complete response (scenario 2). For scenario 1, the best set of sensitivity and specificity was found at 29% difference between middle and initial MNf measurements (sensitivity 36% and specificity 87.0%), while the highest specificity (87.2%) was achieved at 31% reduction of MNf. For scenario 2, the best set of sensitivity and specificity was found again at 29% difference between middle and initial MNf measurements (sensitivity 72.7% and specificity 59.3%), and the highest specificity (59.6%) was found for 31% reduction of MNf (**Table 5**).

Telomerase Activity

As presented in **Table 6**, based on the non-parametric analysis (Kruskal–Wallis), there is no significant difference between

TABLE 2 | Statistical analysis of the mean BNMN and MNf of healthy controls and (A) mCRC/laRC patients at the beginning (before).

	Groups	BN cells scored	Mean ± SE	G	P
BNMN	**Control**	25,000	7.28 ± 1.06		
	mCRC Before	55,000	29.3 ± 9.07	38.00	**<0.001**
	laRC Before	21,000	26.4 ± 4.82	30.15	**<0.001**
MN cells	**Control**	25,000	8.18 ± 1.11		
	mCRC Before	55,000	32.3 ± 9.97	41,1	**<0.001**
	laRC Before	21,000	29.6 ± 5.21	33,76	**<0.001**
CBPI ± SE *(Mean ± SE)*	**Control**	1.29 ± 0.03			
	mCRC Before	1.32 ± 0.004			
	laRC Before	1.36 ± 0.007			

Values in bold font indicate statistically significant differences (P < 0.05) compared with the controls. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

TABLE 3 | Statistical analysis of the mean BNMN and MNf of mCRC patients at the beginning (before), the middle (inter) and at the end (after) of the treatment for all mCRC patients and according to their disease response (progressive disease, stable disease, partial response, and complete response).

mCRC		Sampling Point	BN cells scored	Mean ± SE	G	P
All patients	**BNMN**	**Before**	55,000	29.3 ± 9.07		
		Inter	55,000	19.4 ± 6.86	3.90	**0.04**
		After	55,000	19.6 ± 8.46	3.73	0.05
	MN cells	**Before**	55,000	32.3 ± 9.97		
		Inter	55,000	22.2 ± 8.28	3.65	0.05
		After	55,000	22.6 ± 9.72	3.35	0.06
	CBPI ± SE *(mean ± SE)*	**Before**	1.32 ± 0.004			
		Inter	1.32 ± 0.004			
		After	1.30 ± 0.002			
Progressive disease	**BNMN**	**Before**	13,000	28.8 ± 10.03		
		Inter	13,000	21.3 ± 6.3	2.20	0.14
		After	13,000	20.9 ± 9.66	2.46	0.11
	MN cells	**Before**	13,000	31.3 ± 10.76		
		Inter	13,000	24.6 ± 10.93	1.60	0.20
		After	13,000	23.6 ± 10.92	2.13	0.14
	CBPI ± SE *(mean ± SE)*	**Before**	1.29 ± 0.001			
		Inter	1.32 ± 0.004			
		After	1.31 ± 0.003			
Stable disease	**BNMN**	**Before**	18,000	30,2 ± 8,38		
		Inter	18,000	20,3 ± 6,4	3.77	0.05
		After	18,000	20.1 ± 8.77	3.93	**0.04**
	MN cells	**Before**	18,000	33.1 ± 9.35		
		Inter	18,000	23.1 ± 7.39	3.48	0.06
		After	18,000	23 ± 10.02	3.55	0.06
	CBPI ± SE *(mean ± SE)*	**Before**	1.32 ± 0.003			
		Inter	1.31 ± 0.002			
		After	1.30 ± 0.001			
Partial Response	**BNMN**	**Before**	23,000	29.0 ± 9.54		
		Inter	23,000	17.8 ± 5.98	5.15	**0.02**
		After	23,000	18.7 ± 7.88	4.29	**0.03**
	MN cells	**Before**	23,000	32,5 ± 10.51		
		Inter	23,000	20.6 ± 7.4	5.16	**0.02**
		After	23,000	22.0 ± 9.30	3.94	**0.04**
	CBPI ± SE *(mean ± SE)*	**Before**	1.33 ± 0.006			
		Inter	1.31 ± 0.005			
		After	1.30 ± 0.001			

Values in bold font indicate statistically significant differences (P < 0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

patients' mean TA before the beginning of their therapy (p = 0.256) [progressive disease: 2.1 ± 1.6 (95% CI: 0.4–3.8), stable disease: 1.4 ± 1,7 (95% CI: 0.2–2.6), partial response: 0.8 ± 1.0 (95% CI: 0.1–1.5), complete response: 0.6 ± 0.6 (95% CI: −5.2 to 6,4)], at the middle sampling point (p = 0.072) [progressive disease: 2.8 ± 0.8 (95%CI: 1.9–3.7), stable disease: 1.5 ± 1.3 (95% CI: 0.5–2.4), partial response: 1.1 ± 1.4 (95% CI: 0.1–2.1), complete response: 0.2 ± 0.1 (95% CI: −1.0 to 1.4)] or at the third sampling point (p = 0.096) [progressive disease: 2.5 ± 1.3 (95% CI: 1.1–3.9), stable disease: 1.1 ± 1.2 (95% CI:0.2–2.0), partial response: 1.1 ± 1.3 (95% CI: 0.2–2.0), complete response: 0.9 ± 1.2 (95% CI: −0.2 to 12.0)]. However, as presented in **Table 6** the mean of patients who eventually developed progressive disease, exhibited an overall higher level of TA in relation to all other response groups before the beginning of the therapy. Thereafter, TA was increased and remained elevated during the middle and third sampling points respectively.

DISCUSSION

Driven by the need for effective prognostic and predictive biomarkers in CRC, our study focused on unveiling possible prognostic values of two novel biomarkers (MNf and TA) in PBLs isolated from patients with mCRC and laRC blood samples. Although our findings are hypothesis driven, given the relatively small number of patients we tested, our data provides further proof that MNf is not only significantly increased in CRC [as we have already shown (4)] but it could also serve as a promising biomonitor for mCRC and laRC prognosis. More

TABLE 4 | Statistical analysis of the mean BNMN and MNf of laRC patients at the beginning (before), the end of the treatment (after) and after surgery (surgery) for all laRC patients and according to their disease response (progressive disease, stable disease, partial response and complete response).

laRC		Sampling Point	BN cells scored	Mean ± SE	G	P
All patients	**BNMN**	**Before**	21,000	26.4 ± 4.82		
		After	21,000	16.9 ± 4.00	4,01	**0.04**
		Surgery	12,000	17.4 ± 3.57	3.57	0.06
	MN cells	**Before**	21,000	29.6 ± 5.2		
		After	21,000	19.7 ± 4.7	3.85	**0.04**
		Surgery	12,000	20.8 ± 5.48	3.00	0.08
	CBPI ± SE *(mean ± SE)*	**Before**	1.36 ± 0.007			
		After	1.32 ± 0.003			
		Surgery	1.27 ± 0.003			
Progressive disease	**BNMN**	**Before**	2,000	27.5 ± 6.36		
		After	2,000	21.5 ± 4.95	1.45	0.22
		Surgery	1,000	16.0 ± 5.1	5.80	**0.01**
	MN cells	**Before**	2,000	31.3 ± 10.76		
		After	2,000	23.5 ± 6.36	1.35	0.24
		Surgery	1,000	17.0 ± 5.2	6.42	**0.01**
	CBPI ± SE *(mean ± SE)*	**Before**	1.30 ± 0.001			
		After	1.40 ± 0.005			
		Surgery	1.26 ± 0.002			
Stable disease	**BNMN**	**Before**	5,000	28.7 ± 4.72		
		After	5,000	19.7 ± 3.25	3.25	0.07
		Surgery	3,000	24.0 ± 4.24	0.83	0.36
	MN cells	**Before**	5,000	32.7 ± 4.62		
		After	5,000	23.7 ± 4.72	2.82	0.09
		Surgery	3,000	27.0 ± 2.83	1.09	0.29
	CBPI ± SE *(mean ± SE)*	**Before**	1.30 ± 0.001			
		After	1.40 ± 0.005			
		Surgery	1.26 ± 0.002			
Partial Response	**BNMN**	**Before**	11,000	24.8 ± 4.14		
		After	11,000	14.9 ± 2.93	4.71	**0.02**
		Surgery	6,000	14.4 ± 2.64	5.25	**0.02**
	MN cells	**Before**	11,000	27.8 ± 4.30		
		After	11,000	17.5 ± 3.18	4.50	**0.03**
		Surgery	6,000	17.9 ± 3.18	4.14	**0.04**
	CBPI ± SE *(mean ± SE)*	**Before**	1.35 ± 0.008			
		After	1.30 ± 0.001			
		Surgery	1.30 ± 0.010			
Complete response	**BNMN**	**Before**	3,000	30.0 ± 6.25		
		After	3,000	18.7 ± 5.50	5.05	**0.02**
		Surgery	2,000	22.0 ± 4.24	2.41	0.11
	MN cells	**Before**	3,000	33.7 ± 7.37		
		After	3,000	22.0 ± 6.08	4.77	**0.02**
		Surgery	2,000	22.0 ± 4.24	4.77	**0.02**
	CBPI ± SE *(mean ± SE)*	**Before**	1.45 ± 0.001			
		After	1.32 ± 0.001			
		Surgery	1.22 ± 0.001			

Values in bold font indicate statistically significant differences (P < 0.05) compared with the controls or as indicated. G indicates 2POi ln(Oi/Ei), where 'Oi' is the observed frequency in a cell, 'Ei' is the expected frequency under the null hypothesis, 'ln' denotes the natural logarithm and the sum is taken over all non-empty cells. SE, standard error; BN, binucleated cells (for each patient 1,000 BN cells were scored); BNMN, binucleated cells with micronuclei.

specifically, the present study suggests that a decrease of MNf less than 29% between middle and initial MNf measurements can discriminate between progressive disease from stable/responsive disease with sensitivity of 36% and specificity of 87.0%, while if the threshold is set at 31% reduction of MNf then specificity reaches its highest value (87.2%). On the other hand, if the threshold of decrease is set at 29% for discrimination between stable/progressive and responsive disease then sensitivity reaches 72.7% and specificity 59.3%. **Figure 3** illustrates how MNf may follow a steep decreasing trend in patients with partial/complete response or a shallow decrease in patients with disease progression. These findings are important because early identification of those patients who are more likely to develop progressive disease can allow clinicians to take early decisions on therapy selection. A possible

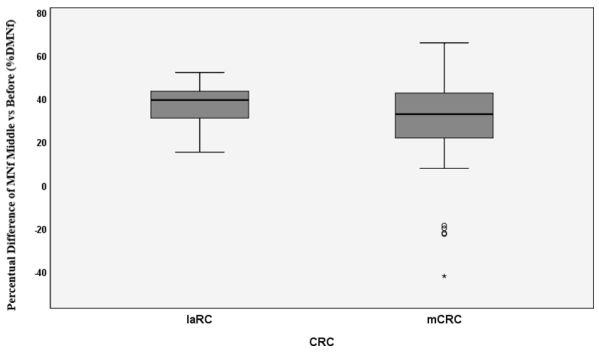

FIGURE 2 | Percentual difference between middle and before measurements of MNf for metastatic colorectal cancer (mCRC) and locally advanced rectal cancer (laRC). * = extreme value as marked in SPSS.

explanation of our findings lies in the fact that MN assay is a sensitive indicator of genomic damages of exogenous and endogenous origin (31, 32), while MNf in PBLs, despite the unknown underlying mechanism, is shown to positively correlate with chromosomal and genomic instability (5), which is one of the pillars in colorectal carcinogenesis. Therefore, the emergence of a resistant cluster of cancer cells against the sensitive "background" can be indirectly identified through MNf. However, as we have already shown (4), and according to our data from this study (not presented here), MNf can be increased after three months of treatment despite favorable disease response in case they are treated with combined treatment of FOLFIRI plus any biologic agent and then present a significant decrease at the end of the treatment. Therefore, the use of MNf as a prognostic biomarker in such patients may not be appropriate, and special caution is suggested for its use. Nonetheless, it could be used as a biomonitoring tool of cancer load. As for TA, even though the

TABLE 5 | Sensitivity and specificity values for three different cutoff points of percentage difference of MNf.

Limit	Outcome							
	Prog. (N_T = 15)		S.D./P.R./C.R. (N_T = 61)		Prog./S.D. (N_T = 38)		P.R./C.R. (N_T = 38)	
	N	%	N	%	N	%	N	%
≤33%	9	25.0%	27	75.0%	20	55.6%	16	44.4%
>33%	6	15.0%	34	85.0%	18	45.0%	22	55.0%
≤31%	9	31.0%	20	69.0%	19	65.5%	10	34.5%
>31%	6	12.8%	41	87.2%	19	40.4%	28	59.6%
≤29%	8	36.4%	14	63.6%	16	72.7%	6	27.3%
>29%	7	13.0%	47	87.0%	22	40.7%	32	59.3%

It can be seen that when the outcome is progressive disease vs stable/ partial/complete response, the best set of sensitivity–specificity is found at 29% difference between middle and initial MNf measurements (sensitivity 36.4% and specificity 87.0%). The highest specificity (87.2%) was found for 31% reduction of MNf. When the outcome was set between stable/progressive disease vs partial/complete response, the best set of sensitivity and specificity variables was found for 29% difference (sensitivity 72.7% and specificity 59.3%). NT, total number of patients; Prog., progression; S.D., stable disease; P.R., partial response; C.R., complete response.

TABLE 6 | Telomerase activity measured at three time points, before (before the initiation of the therapy), middle (at the middle of therapy for mCRC and at the end of therapy for laRC), and after (at the end of the therapy for mCRC and after surgery for laRC) is presented for all CRC cases.

Telomerase Activity		n	Mean	SD	95%		Minimum	Maximum	p
					LL	UL			
Before	**Progression**	6	2.1	1.6	0.4	3.8	0.1	3.7	0.256
	Stable disease	10	1.4	1.7	0.2	2.6	0.0	3.8	
	Partial response	10	0.8	1.0	0.1	1.5	0.0	2.4	
	Complete Response	2	0.6	0.6	–5.2	6.4	0.2	1.1	
	Total	28	1.3	1.4	0.7	1.8	0.0	3.8	
Middle	**Progression**	6	2.8	0.8	1.9	3.7	1.7	3.7	0.072
	Stable disease	10	1.5	1.3	0.5	2.4	0.0	3.5	
	Partial response	10	1.1	1.4	0.1	2.1	0.0	3.2	
	Complete Response	2	0.2	0.1	–1.0	1.4	0.1	0.3	
	Total	28	1.5	1.4	1.0	2.1	0.0	3.7	
After	**Progression**	6	2.5	1.3	1.1	3.9	0.1	3.6	0.096
	Stable disease	10	1.1	1.2	0.2	2.0	0.0	3.3	
	Partial response	10	1.1	1.3	0.2	2.0	0.0	3.3	
	Complete Response	2	0.9	1.2	–10.2	12.0	0.0	1.8	
	Total	28	1.4	1.3	0.9	1.9	0.0	3.6	

p-values were estimated with Kruskal–Wallis test. n, number of patients.

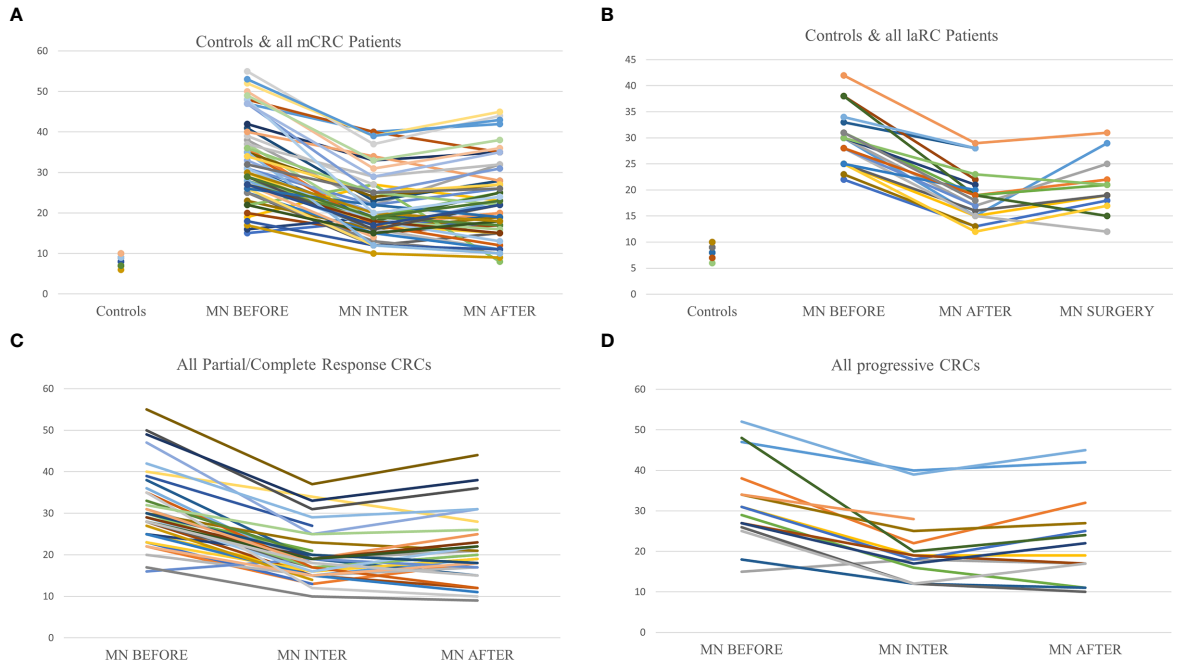

FIGURE 3 | Graphic representation of MNf in controls and the trends between sampling points for every patient. **(A)** Presentation of MNf trend for controls and all mCRC patients; **(B)** presentation of MNf trend for controls and laRC patients; **(C)** presentation of MNf trend for all patients (mCRC and laRC) with partial or complete response; **(D)** presentation of MNf trend for all patients (mCRC and laRC) with progressive disease.

number of patients to which TA was evaluated is rather small, our results indicate its potential in CRC prognosis suggesting the need for future studies with greater patient sets. Despite the fact that statistical analysis between each response group did not reach statistical significance, our data indicate that TA is relatively higher in patients with progressive disease than those with partial or complete response at all sampling points. Moreover, in patients with progressive disease, an increase of TA is observed in the middle of their therapy, suggesting that patients who are more likely to develop progressive disease are more likely to have upregulated TA at the middle of their therapy. This can be explained by numerous studies that have identified telomerase as a key target of multiple carcinogenic pathways such as the PI3K/AKT/mTOR, the RAS/RAF/MEK/ERK 1/2, the JAK/STAT, and the JAK/PI3K/AKT/HSP90/mTORC1 (33–38). An interesting finding highlighting the complexity of hTERT regulation is that EGFR-mediated MAPK signaling attenuates Groucho-mediated gene repression, establishing a node for crosstalk between the EGFR, Notch, WNT, and TGF-β signaling pathways (39). A graphical presentation of the aforementioned mechanisms is shown in **Figure 4**. Unfortunately, since our primary objective was to investigate possible prognostic significance of these biomarkers, we did not examine any de-regulation of the aforementioned pathways.

Finally, in order to thoroughly understand and interpret our results, it is important to know how these biomarkers are affected by the different therapeutic agents used in our study. To begin with, FOLFOX, FOLFIRI, and FOLFOXIRI regimens are the three principal first-line therapeutic regimens administered for stage IV CRC according to HeSMO guidelines (20). They share two components, folinic acid (FA) and 5-fluorouracil (5-FU), while oxaliplatin and irinotecan are the compounds that differentiate the first two respectively or if combined make the later. CAPOX on the other hand, which along with FOLFOX is primarily used to treat laCRC (21), contains capecitabine and oxaliplatin. Based on bibliographic evidence validating the effect of these agents upon MNf and TA, it is evident that there is no universal effect. Overall, it is reported that 5-FU, capecitabine, oxaliplatin, and irinotecan increase BNMN (40–42), while FA significantly reduces it (43). Interestingly, as mentioned earlier, our data provides further proof that irinotecan and therefore FOLFIRI may increase MNf. However, for the most part, little data exists regarding any alterations of TA concomitant to administration of these substances. According to Akiyama et al., TA was decreased in human hematopoietic cancer cell lines, Daudi and U937, treated with irinotecan (44). However, there is no data suggesting any possible alterations of TA in CRC cell lines. Chung et al., using the HCT116 and DLD1 CRC cell lines, demonstrated a decreased TA concurrent to 5-FU administration possibly *via* STAT3 inhibition (a potent activator of hTERT promoter) (45). However, to the best of our knowledge, to date there is no study available evaluating the effect of the chemotherapeutic regimens of FOLFOX, FOLFIRI, FOLFOXIRI, or CAPOX on any of our biomarkers in this study. Given the great number of our patients treated with some kind of biologic agent (bevacizumab, cetuximab, panitumumab, or

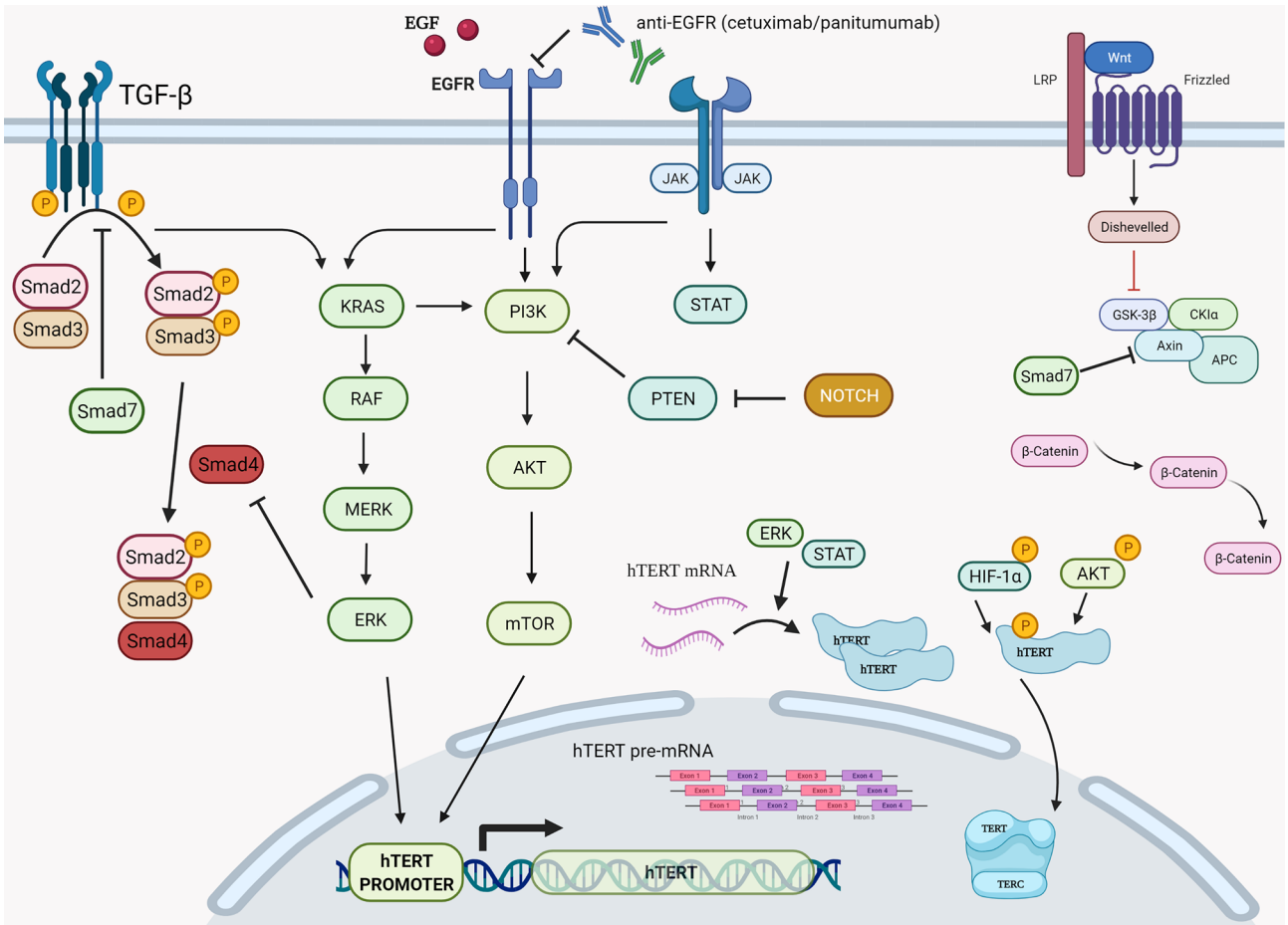

FIGURE 4 | Graphical presentation of the various cellular cascades implicated in hTERT expression, telomerase activity, and telomere length. During transcription of hTERT gene, positive regulation is exerted *via* the PI3K/AKT/mTOR, the RAS/RAF/MEK/ERK 1/2, and the JAK/STAT pathways primarily on hTERT's promoter. Another step of positive regulation is found during the translation and post translation phase of hTERT mRNA since the RAS/RAF/MEK/ERK pathway is described to regulate hTERT mRNA translation, while the PI3K/AKT/mTOR pathway seems to be involved post-translationally *via* direct phosphorylation of hTERT by AKT and HIF-1α. RAS, rat sarcoma; RAF, rapidly accelerated fibrosarcoma; MEK also known as MAP2K, MAPKK, mitogen-activated protein kinase kinase; ERK, also known as MAPK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PKB, also known as Akt, protein kinase B; Mtor, mechanistic target of rapamycin kinase; HIF-1α, hypoxia-inducible factor-1α. Created with BioRender.com.

aflibercept), we expanded our research to include them as well. The only published data focusing on MNf comes from an *in vivo* cytogenetic assay performed in male Wistar rats, where cetuximab did not elicit any genotoxic effects (46). However, the results should be considered of limited value due to the lack of immunoreactivity of Cetuximab with rat tissues. To conclude, despite critical advances in most aspects of CRC management, it is indisputably one of the most important burdens of global health due to the related increased morbidity and mortality. Metastatic CRC, the final stage of CRC, remains a true challenge, not only for researchers and clinicians, but also for the socioeconomic system. This is because its inherent biologic complexity and diversity make it difficult to implement a universal approach in designing effective therapeutic and study protocols. On the contrary, even though laRC is a rather favorable type of CRC due to the absence of distant metastases, there still is a metastatic potential. Therefore, early recognition of chemoresistance is crucial. Our study made it possible not only to recognize possible prognostic significance at an early stage of therapeutic management for two novel biomarkers (MNf and TA), but also to suggest a relative threshold for MNf as a discrimination point between progressive and stable/responsive disease. However, the results of the current study should be interpreted with caution due to the limitations of the protocol (relatively small number of cases, different systemic treatment, different mutations subtypes, not randomized manner *etc.*) and could mainly serve as hypothesis generating study for further evaluation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Committee at the University Hospital of Heraklion. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TN conducted the experiments, interpreted the data, performed the analysis, and wrote the manuscript. EV conducted experiments. PS performed the analysis and interpreted the data. AA performed the statistical analysis, interpreted the data, and wrote the manuscript. AB conducted experiments. NR conducted experiments. JS provided blood samples, raised funds, edited, and critically revised the manuscript. AT raised funds, edited and critically revised the manuscript. JT conceived and designed the study, raised funds, edited, and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.