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## Telomere and Centromere Staining Followed by M-FISH Improves Diagnosis of Chromosomal Instability and Its Clinical Utility

Radhia M'kacher, Bruno Colicchio, Claire Borie, Steffen Junker, Valentine Marquet, Leonhard Heidingsfelder, Kevin Soehnen, Wala Najjar, William M. Hempel, Noufissa Oudrhiri, et al.

► **To cite this version:**

Radhia M'kacher, Bruno Colicchio, Claire Borie, Steffen Junker, Valentine Marquet, et al.. Telomere and Centromere Staining Followed by M-FISH Improves Diagnosis of Chromosomal Instability and Its Clinical Utility. *Genes*, 2020, 11 (5), pp.475. 10.3390/genes11050475 . hal-03753192

**HAL Id: hal-03753192**

**<https://hal.science/hal-03753192>**

Submitted on 17 Aug 2022

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1 Article

2 **Telomere and centromere staining followed by**  
3 **M-FISH improves diagnosis of chromosomal**  
4 **instability and its clinical utility**

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38 Received: date; Accepted: date; Published: date

39 **Abstract:**

**40 Background:**

41 Chromosomal instability is the most important predictive and prognostic biomarker of the  
42 exposure of populations and patients to genotoxic agents. One relevant marker is the presence of  
43 dicentric chromosomes. The appearance of such chromosomes is associated to telomere  
44 dysfunction, leading to cancer progression and poor clinical outcome. Here, we present improved  
45 methods for the detection of telomere dysfunction and the identification of dicentric chromosomes,  
46 two driving forces of chromosomal instability.

**47 Materials and methods:**

48 Peripheral blood samples from a cohort of 50 cancer patients, 44 with miscellaneous genetic  
49 syndromes, and 100 healthy donors were examined. Sequential analysis using telomere and  
50 centromere staining followed by M-FISH (TC+M-FISH) was performed to characterize telomere  
51 dysfunction and chromosomal aberrations.

**52 Results:**

53 Significant telomere shortening was found in the peripheral blood cells of patients with cancer and  
54 genetic syndromes in comparison to similar age-matched healthy donors ( $p < 10^{-7}$  and  $p < 10^{-10}$   
55 respectively). Significantly higher frequencies of telomere loss and telomere deletion were detected  
56 in the blood ( $p < 10^{-13}$  and  $p < 10^{-8}$  for cancer patients;  $10^{-13}$  and  $10^{-2}$  for patients with genetic  
57 syndromes as compared to healthy controls). We assessed the potency of our technique against  
58 conventional cytogenetics for the detection of dicentric chromosomes by subjecting metaphase  
59 preparations from patients with cancer and genetic syndromes to both approaches, TC+M-FISH  
60 sequential analysis and standard cytogenetics in parallel. We identified dicentric chromosomes in  
61 28/50 cancer patients and 21/44 genetic syndrome patients using our TC+M-FISH sequential  
62 analysis, but only 7/50 and 12/44, respectively, using standard cytogenetics. We ascribe this  
63 discrepancy to the identification of the unique configuration of dicentric chromosomes. We  
64 observed significantly higher frequencies of telomere loss and deletion in patients with dicentric  
65 chromosomes ( $p < 10^{-4}$ ).

**66 Conclusion:**

67 We demonstrate that the TC+M-FISH sequential analysis is superior to classical cytogenetics for the  
68 detection of dicentric chromosomes and chromosomal instability. Moreover, our approach is a  
69 relatively simple but useful tool for documenting telomere dysfunction. We suggest our technique  
70 to become a standard additional diagnostic tool in the clinics.

71

72 **Keywords:** telomere, centromere, dicentric chromosome, chromosomal instability, cancer, genetic  
73 syndrome

74

**75 1. Introduction**

76 Chromosomal instability is defined as the progressive accumulation of numerical and  
77 structural chromosomal aberrations and drives cancer initiation and evolution [1, 2]. Chromosomal  
78 instability has proven to be an essential biomarker for patients with cancer, inflammatory diseases,  
79 and individuals in otherwise healthy populations exposed to genotoxic agents, as well as their  
80 progeny [3, 4]. Ample evidence acquired during the past decades has demonstrated the predictive

81 and prognostic value of chromosomal instability as a biomarker for treatment response and clinical  
82 outcomes of these populations [5-10].

83 Substantial progress has been achieved in the detection and identification of chromosomal  
84 instability. Conventional karyotyping involving *e.g.* Giemsa banding (G-banding) or inverted DAPI  
85 (4',6'-diamidino-2-phenylindole) staining is still the most precise and reliable technique used in  
86 research and clinical settings [11]. Despite its limitations in the detection of aberrations, *i.e.* less than  
87 one megabase, as well as karyotype heterogeneity, conventional karyotyping has proven to be an  
88 important tool for clinical diagnosis, especially among onco-hematology patients [12-14] and those  
89 with genetic disorders [15]. The introduction of fluorescence in situ hybridization (FISH) in  
90 cytogenetic analyses has immensely improved the detection of numerical and structural aberrations  
91 in metaphases, as well as interphases [16]. However, genomic analysis (NGS or micro-arrays) has  
92 lately made a sensational entrance into the clinical field [17]. Nevertheless, these approaches have  
93 their shortcomings in that they fail to detect the level of karyotype heterogeneity, as well as  
94 chromosomal instability involving repeated sequences, telomeres, and centromeres [18].

95 Among the three main pathways of chromosomal instability, *i.e.* random breakage, telomere  
96 fusion, and centromere fission, the last two are generally underestimated using standard cytogenetic  
97 techniques [19].

98 Telomeres protect the ends of chromosomes, securing genome stability and integrity.  
99 Dysfunction of the telomere nucleoprotein complex can expose free chromosome ends to the DNA  
100 double-strand break (DSB) repair machinery, leading to telomere fusion and dicentric chromosome  
101 formation. Thus, the consequences of telomere loss or dysfunction can promote chromosomal  
102 instability, leading to the progression of malignant cancer and poor clinical outcome [14, 20].

103 Centromeres are essential to eukaryotic biology by orchestrating the transmission of the  
104 genome during cell division. However, although comprising 2 to 5% of the human genome, they are  
105 nevertheless still largely a genetic black box [21]. Chromosomal breakpoints at (peri) centromere  
106 regions are found in several tumors and are associated with chromosomal instability [22].  
107 Mechanisms leading to centromere breaks are still not well understood [18]. However,  
108 pericentromeric instability and breakage is evident in ICF syndrome, caused by defects in DNA  
109 methylation due to the impairment of DNA methyltransferase activity [23, 24].

110 Various methods have allowed the identification of several aberrations involving centromeres  
111 and telomeres in patients and populations exposed to genotoxic agents. We recently demonstrated  
112 that the introduction of telomere and centromere (TC) staining followed by M-FISH (TC+M-FISH)  
113 not only renders the analysis of chromosomal aberrations more efficient and robust, but also permits  
114 the detection of specific configurations of dicentric chromosomes and their persistence, highlighting  
115 the importance of these configurations [25, 26].

116 Here, we demonstrate that combining inverse DAPI, and TC+M-FISH allows enhanced  
117 detection of chromosomal aberrations and, in particular, that of dicentric chromosomes. We provide  
118 validation of this approach in the detection of chromosomal instability. Our technique is readily  
119 applicable to the research lab and the clinic.  
120

## 121 2. Materials and Methods

### 122 2.1. Materials

123 Peripheral blood lymphocytes were obtained from a cohort of 50 patients with hematopoietic  
124 malignancies and 44 with genetic disorders (Table 1). One hundred healthy donors were used as a  
125 control. Cytogenetic preparations were produced from patients and healthy donors. TC+M-FISH  
126 was performed to characterize telomere dysfunction and chromosomal aberrations. The collection of  
127 blood samples from patients and donors was approved by the Ethics Committee of Gustave Roussy  
128 Cancer Campus University Paris Saclay (approval number 97-06).

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Table1: Characteristics of Cancer patients and genetic disorders patients

Characteristics	No. of Patients
<b>Cancer patients</b>	50
Male	32
Age (years)	45
Type	
Hodgkin lymphoma	18
Non Hodgkin lymphoma	15
Mantel Cell lymphoma	8
B-cell prolymphocytic leukemia	2
Myelodysplasia syndrome	4
Other	5
<b>Genetic syndrome</b>	44
Male	27
Age (years)	32
Type	
Turner syndrome	9
Down syndrome	4
Li-Fraumeni	2
Telomerepathies	2
Other	17

143

## 144 2.2. Methods:

### 145 2.2.1. Preparation of Metaphase Spreads

146 Peripheral blood lymphocytes were cultured in RPMI1640 and exposed to colcemid (0.1 µg/mL)  
147 (Gibco KaryoMAX,) for 2 h at 37°C, 5% CO<sub>2</sub>, in a humidified atmosphere to arrest dividing cells in  
148 metaphase. After harvesting the cells, they were centrifuged for 7 min at 1,400 RPM at room  
149 temperature, the supernatant removed, the cell pellet was re-suspended in a solution of warm (37°C)  
150 0.075 M potassium chloride (KCl) (Merck, New Jersey, US) and incubated for 20 min in a 37 °C water  
151 bath (hypotonic shock). The cells were pre-fixed by adding approximately five drops of fixative (3:1  
152 ethanol/acetic acid) to each tube under agitation and the tubes centrifuged for 7 min at 1,400 RPM at

153 room temperature. The supernatant was removed and the cells suspended in the fixative solution  
154 and centrifuged using the same parameters. After two additional rounds of these fixation steps, the  
155 cells were stored in the fixative solution at 4°C overnight and the metaphases spread on cold wet  
156 slides the next day. The slides were dried overnight at room temperature and stored at -20 °C until  
157 further use.

#### 158 2.2.2. TC+M-FISH:

159 Telomeres and centromeres were stained by Q-FISH with a Cy-3-labelled PNA probe specific  
160 for TTAGGG for telomeres and a FITC-labeled probe specific for centromere sequences (obtained  
161 from Eurogentec, Leige, Belgique), as described in M'kacher *et al.* [25, 27]. Briefly, slides were  
162 washed with 1X PBS and fixed with 4% formaldehyde at room temperature. After rinsing three  
163 times with PBS, the slides were treated with pepsin (0.5 mg/ml) at 37°C for 5 min. After rinsing three  
164 times with PBS, the slides were sequentially dehydrated with 50%, 70%, and 100% ethanol and  
165 air-dried. The telomere and centromere probes were added to the slides and subsequently  
166 denatured on a hot plate at 80°C for 3 min and then incubated in the dark for 1 h at room  
167 temperature. The slides were subsequently rinsed with 70% formamide/10 mM Tris pH 7.2 three  
168 times during 15 min and then in 50 mM Tris pH7.2/150 mM NaCl pH 7.5/0.05% Tween-20 (3 x 5min).  
169 After a final rinse in PBS, the slides were counterstained with DAPI and mounted in PPD at the  
170 appropriate pH. The slides were captured and analyzed by M-FISH (MFISH 24XCyte, Metasystems,  
171 Altussheim, Germany) [25, 26].

#### 172 2.2.3. Telomere Quantification

173 Two approaches were developed for the quantification of telomere length using TeloScore  
174 software. The first consisted of the quantification of telomeres in interphase cells, permitting the  
175 investigation of intercellular variation in a large number of scored cells. Quantitative image  
176 acquisition was performed using MetaCyte software (MetaSystem, version 3.9.1, Altussheim,  
177 Germany). The exposure and gain settings remained constant between captures. The analysis was  
178 performed using TeloScore Software (Cell Environment, Paris France). The mean fluorescence  
179 intensity (FI) of telomeres was automatically quantified and analyzed in 10,000 nuclei on each slide.  
180 The experiments were performed on triplicate slides. The second approach consisted of quantifying  
181 telomere length in metaphases using ChromoScore Software (Cell Environment, Paris France). The  
182 images of metaphases were captured using automated acquisition module Autocapt software  
183 (MetaSystems, version 3.9.1) and a ZEISS Plan-Apochromat 63x/1.40 oil and CoolCube 1 Digital  
184 High-Resolution CCD Camera with constant settings for exposure and gain. The telomere lengths of  
185 individual chromosomes, as well as the mean telomere length of metaphases, were measured.

186 Telomere length, measured as the mean fluorescence intensity (FI), strongly correlated with  
187 telomere length measured by Southern-blot analysis using the telomeric restriction fragment (TRF)  
188 ( $R^2 = 0.721$  and  $p = 2.128e-8$ ). The mean telomere length is expressed in kb.

#### 189 2.2.4. Scoring of telomeres and chromosomal aberrations.

190 For each patient, telomere and chromosomal aberrations were analyzed on an average of 100  
191 metaphases. Telomere aberrations were assessed after telomere and centromere staining: (i)  
192 telomere loss was defined as a signal-free end at a single chromatid, an aberration that leads to  
193 telomere-end-fusion and breakage-fusion-bridge cycles[28]; (ii) telomere doublets or telomere  
194 fragility were defined as more than one telomere signal at a single arm, an aberration signaling  
195 inadequate telomere replication and the dysfunction of shelterin proteins[29, 30]; (iii) telomere  
196 deletion was defined as the loss of two telomere signals on the same arm, an aberration considered  
197 to represent double strand breaks, leading to the activation of DNA repair mechanisms. Automatic  
198 scoring of these aberrations was performed using ChromoScore software (Cell Environment, Paris,  
199 France) and an operator validated and excluded the false aberrations. Ikaros software was used for  
200 the classification of chromosomes following conventional cytogenetic G or R-Banding and Isis  
201 software was used after M-FISH. In addition to the scoring of translocations, insertions and

202 deletions, TC+M-FISH allowed the scoring of dicentric chromosomes, centric rings, and various  
203 types of acentric chromosomes.

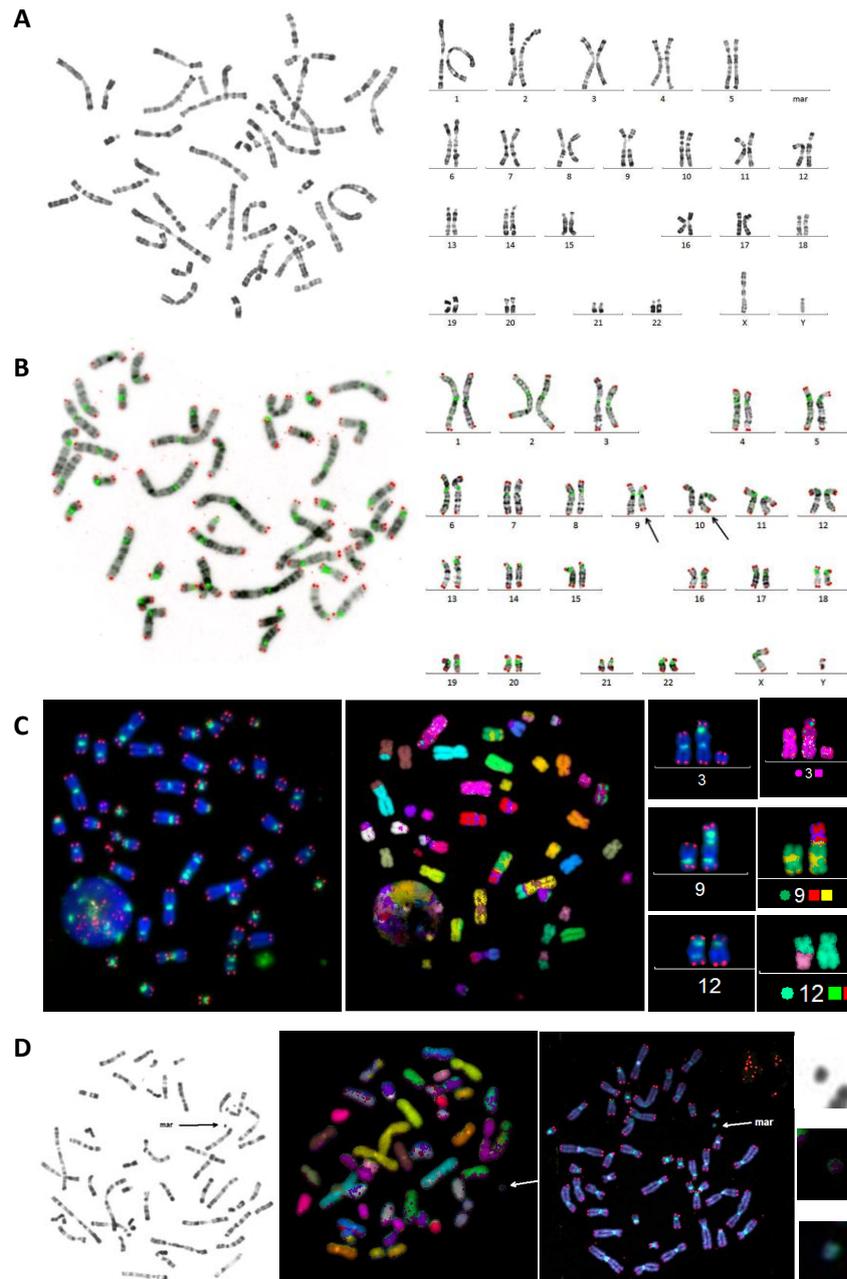
#### 204 2.2.4. Statistical Analysis

205 Data were analyzed by Wilcoxon-Mann-Whitney rank sum test or the Kruskal-Wallis  
206 non-parametric test. A p value < 0.05 was considered statistically significant.

### 207 3. Results

#### 208 3.1-Improved identification of chromosomal aberrations after TC staining

209 Identification of chromosomal aberrations using conventional cytogenetic techniques is based  
210 on chromosome morphology and G-Banding or inverted DAPI staining. These approaches allow the  
211 identification of chromosomes according to their size and specific banding patterns (Figure 1A). The  
212 introduction of centromere staining allowed visualization of the centromeric regions and, as a result,  
213 the identification of dicentric chromosomes, centric rings, and acentric fragments. Furthermore,  
214 telomere staining made it possible to identify chromosome ends and improved the identification of  
215 chromosome territories, particularly in the case of overlapping chromosomes, and thus the detection  
216 of chromosomal aberrations. TC staining also made it possible to eliminate false-positive and  
217 false-negative aberrations, which are impossible to detect with conventional cytogenetics. In  
218 addition, TC staining improved classification of chromosomes related not only to their size, but also  
219 to the size of the p and q arms, respectively. Moreover, inverted DAPI, similar to GTG banding,  
220 improved the efficiency of the identification of chromosomes and the detection of their aberrations  
221 (Figure 1B). TC staining also improved the detection of dicentric chromosomes with centromeres in  
222 close proximity or in contact with the telomeres, as well as cases of the two centromeres being in  
223 close proximity (Figure 1C). It is now possible to score and distinguish very small centric and  
224 acentric rings more accurately. Hitherto, this configuration has been extremely difficult to detect  
225 using conventional and molecular approaches (Figure 1C-D).

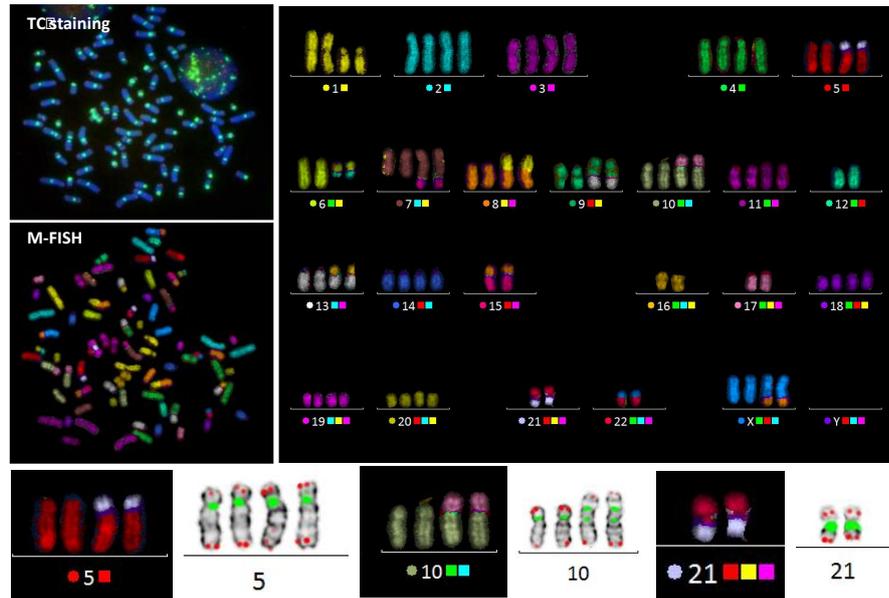


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 227 Figure 1. Cytogenetic detection of chromosomal aberrations. (A) G-banding, based on the morphological criteria of  
 228 chromosomes, was the first and widely used technique for clinical cytogenetics. (B) Telomere (red) and centromere (green)  
 229 staining allows the reliable classification of chromosomes and the precise detection of chromosomal aberrations, such as  
 230  $t(9;10)(q,q)$ . This reciprocal translocation involved the telomere region of chromosome 10. (C) The precise detection of the  
 231 centromeric region leads to the reliable detection of dicentric chromosomes, especially if both centromeres are very close. The  
 232 M-FISH technique does not stain the centromeric region. (D) The detection of a centric ring in circulating lymphocytes of a  
 233 patient with a genetic syndrome by TC staining. This centric ring was undetectable by G-banding or M-FISH

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In cases of a complex karyotype, TC+M-FISH allows enhanced visualization of repeated sequences (telomeres and centromeres), which are undetectable using M-FISH on its own, and is also a more reliable method for the detection of chromosomal aberrations. Dicentric chromosomes may be mistaken for translocations using M-FISH alone. The detection of dicentric chromosomes with a specific configuration (*i.e.* both centromeres in close proximity) can be achieved using TC staining followed by M-FISH technique. The power of this approach to identify characteristic

241 dicentric chromosomes in the blood cells of an acute lymphoblastic leukemia patient with both  
 242 centromeres in intimate contact is demonstrated in Figure 2.  
 243



244

245 Figure 2. TC+M-FISH used to detect a complex karyotype in a case of acute lymphoblastic leukemia. This approach  
 246 makes it possible to detect not only the translocation and complex exchange but also the presence of a specific configuration  
 247 of dicentric chromosomes with both the centromeres very close to each other.

### 248 3.2.-Telomere instability detected by TC+M-FISH staining

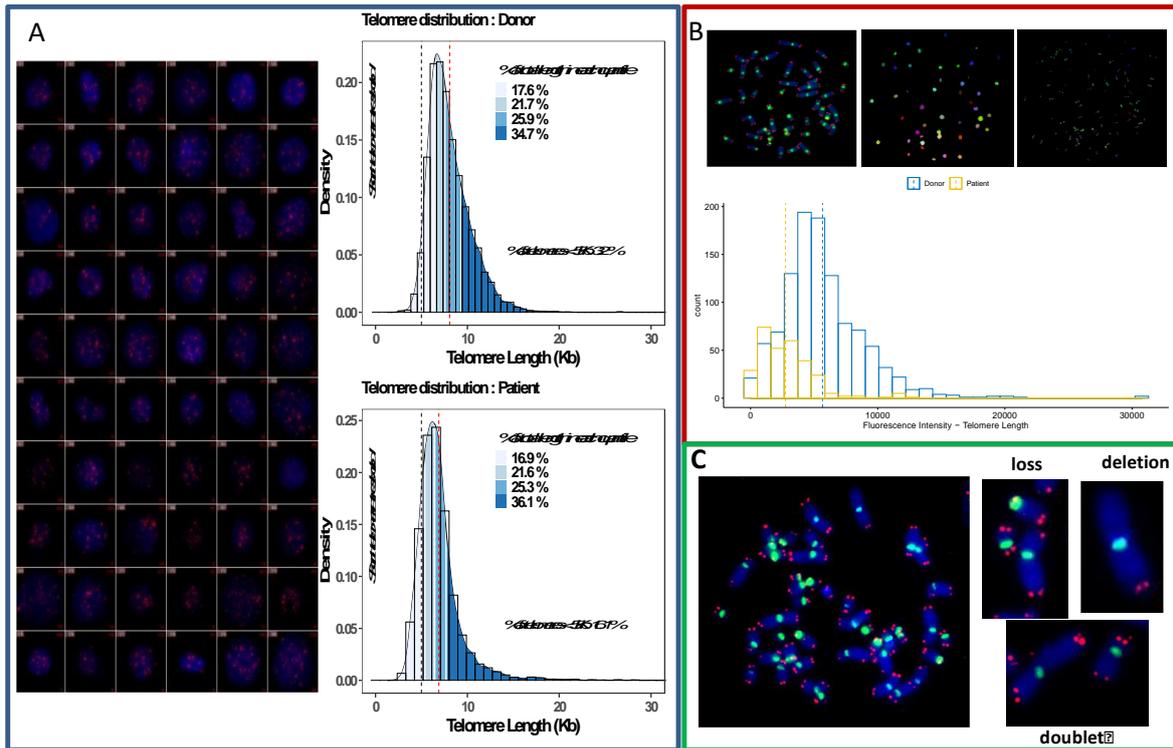
249 Telomere instability is defined by telomere shortening and/or telomere dysfunction (uncapped  
 250 or damaged telomeres) and is considered to be an important mechanism underlying chromosomal  
 251 instability. Thus, telomere instability may be a key player in the process of oncogenesis. However,  
 252 the absence of a proper technique to detect telomere instability adapted to the clinical routine has  
 253 hitherto led to global underestimation of its role. The introduction of TC staining in the cytogenetics  
 254 for clinical investigation now permits the assessment of telomere length and instability.

255 After TC staining, the quantification of telomere length can be performed in interphase nuclei,  
 256 as well as in metaphases.

257 Global quantification of the fluorescence intensity of the DNA strand of telomeric regions in  
 258 nuclei permits the detection of not only the mean telomere length, but also the inter-cellular  
 259 variation and proportion of cells with drastic telomere shortening (Figure 3A).

260 The quantification of telomere length can be performed on metaphases, allowing measurement  
 261 of the intensity of the fluorescence of each telomere signal. This approach allows the analysis of the  
 262 intra-chromosomal variation and heterogeneity of telomere signals in metaphases (Figure 3B).

263 TC staining is a unique technique that permits the analysis of telomere loss, telomere deletion,  
 264 and the formation of telomere doublets for each chromosome, offering the possibility to assess clonal  
 265 telomere aberrations after the classification of chromosomes has been performed in metaphases  
 266 (Figure 3C).



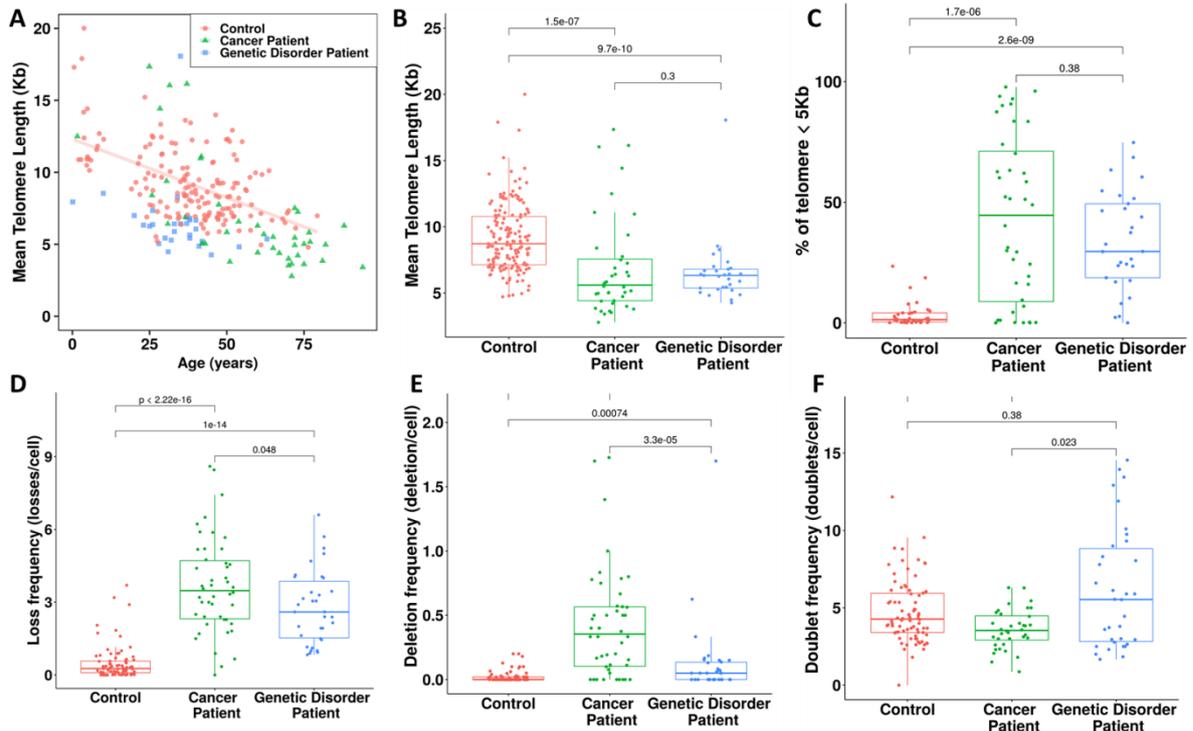
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Figure 3. Quantification of telomere length and telomere dysfunction. (A) Global quantification of telomere length in the nuclei allows its evaluation in a large number of cells and the analysis of the intercellular heterogeneity of telomere length. An example of the quantification of the telomere length of a healthy donor and a cancer patient show the difference in the frequency of cells with drastic telomere shortening. Mean telomere length is not always the appropriate indicator. (B) The use of cytogenetic slides in the quantification of telomere length offers the possibility to detect the telomere sequences in metaphases, the quantification of telomeres, and the assessment intrachromosomal variations in telomere length. (C) The use of metaphases permits the detection of telomere aberrations: telomere loss, telomere deletion (the loss of two telomeres in the same arm), and the formation of telomere doublets. These telomere aberrations are related to telomere dysfunction.

277 **3.3. Validation of the concept**

278 **3.3.1. Telomere dysfunction**

279 Quantification of telomere length using this approach showed the dependence of telomere length  
280 on age in healthy donors, with high inter-individual variation. The natural mean decrease of telomere  
281 length was 79 bp/year (Figure 4A). Patients with cancer or genetic disorders showed a significant  
282 reduction in mean telomere length relative to controls ( $p < 10^{-7}$  for cancer patients;  $p < 10^{-10}$  for patients  
283 with genetic disorders) (Figure 4B). This difference in telomere length between cancer patients and those  
284 with genetic disorders and controls increased when we analyzed the frequency of cells with major  
285 telomere shortening ( $< 5$  kb) ( $p < 10^{-13}$  for cancer patients and those with genetic disorders) (Figure 4C).

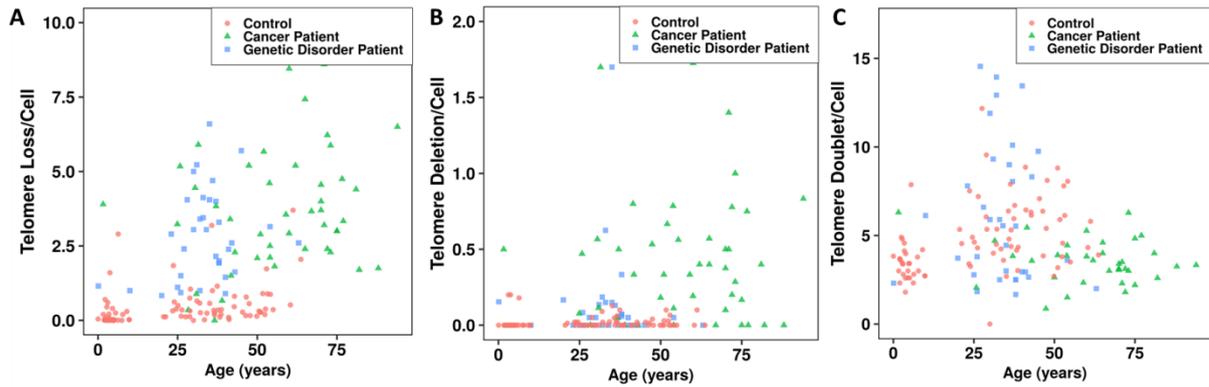


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Figure 4: Telomere dysfunction in cancer patients and those with genetic syndromes. (A) The telomere length of healthy donors is age dependent, with a mean decrease of 79 bp per year. In cancer patients and those with genetic disorders, there was no significant correlation between telomere length and age. High individual variation was observed in telomere length of healthy donors, cancer patients, and genetic disorder patients. (B) Cancer patients and those with genetic syndromes show significantly shorter telomeres than healthy donors. (C) Analysis of the frequency of cells with short telomeres (< 5 kb) revealed a significant difference between cancer patients and those with genetic disorders and healthy donors. (D) The frequency of telomere loss, the major telomere aberration that leads to telomere fusion and chromosomal instability, was significantly higher in cancer patients and those with genetic syndromes than healthy donors. (E) Similarly, the frequency of telomere deletions was significantly higher in cancer patients and those with genetic disorders than healthy donors. (F) There was no significant difference between telomere doublet formation in patients with genetic disorders and healthy donors. Healthy donors are represented by red circles, cancer patients by blue triangles, and cancer with genetic disorder by green triangles.

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The analysis of telomere loss, deletion, and doublet formation in healthy donors demonstrated that these telomere aberrations are age independent (Figure 5). In cancer patients and those with genetic disorders, higher frequencies of telomere loss ( $p < 10^{-13}$ ;  $p < 10^{-13}$ , respectively) (Figure 4D), telomere deletions ( $p < 10^{-8}$ ;  $p < 10^{-2}$ , respectively) (Figure 4E), and age independence (Figure 5A-B) were observed than in healthy donors. However, cancer patients showed significantly less telomere doublet formation than healthy controls and patients with genetic disorders ( $p = 0.01$  and  $p = 0.02$ , respectively) (Figure 4F, Figure 5C). There was no significant difference between the frequency of telomere doublet formation in patients with genetic syndromes and healthy donors (Figure 4F, Figure 5C).

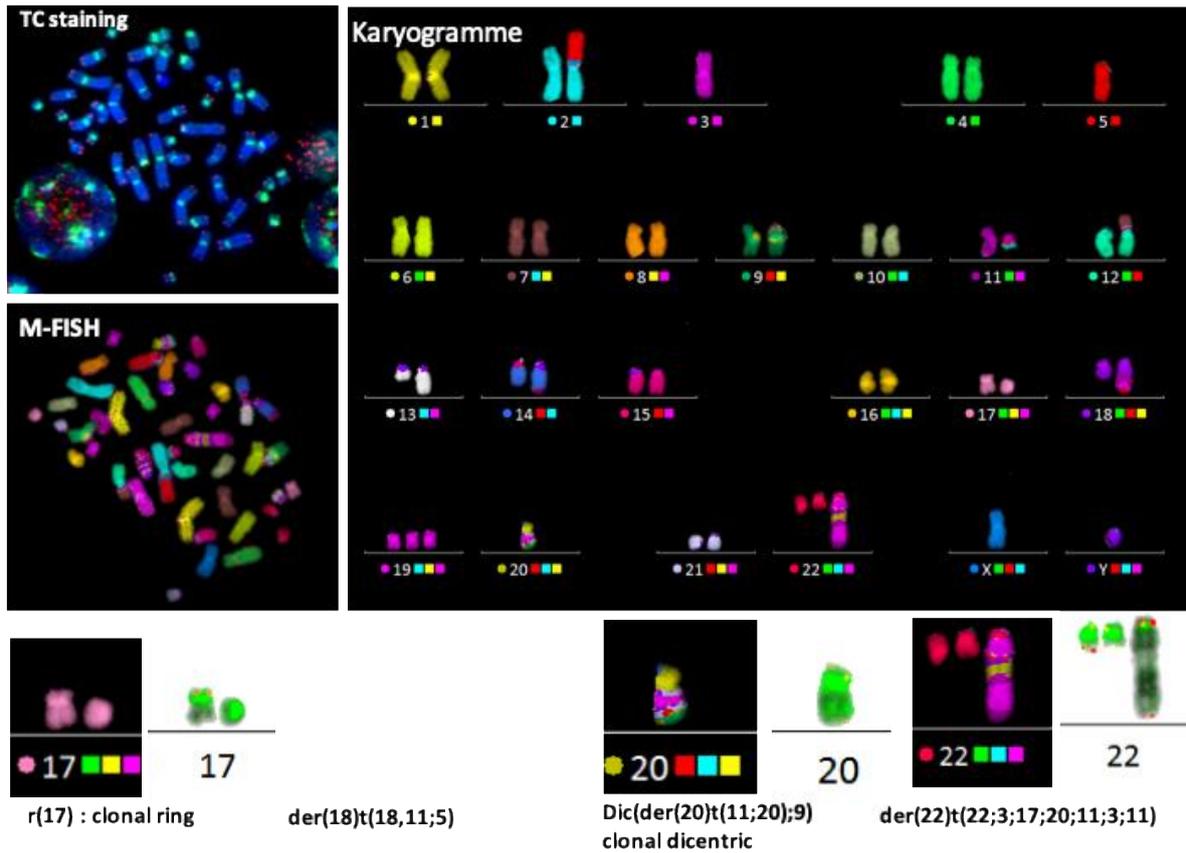


307  
 308 Figure 5. Variation of telomere dysfunction with age in healthy donors, cancer patients, and patients with genetic  
 309 disorders: telomere dysfunction is relatively independent of age in all groups: (A) telomere loss, (B)  
 310 telomere deletion, and (C) telomere doublet formation.

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### 312 3.3.2. Detection of dicentric chromosomes

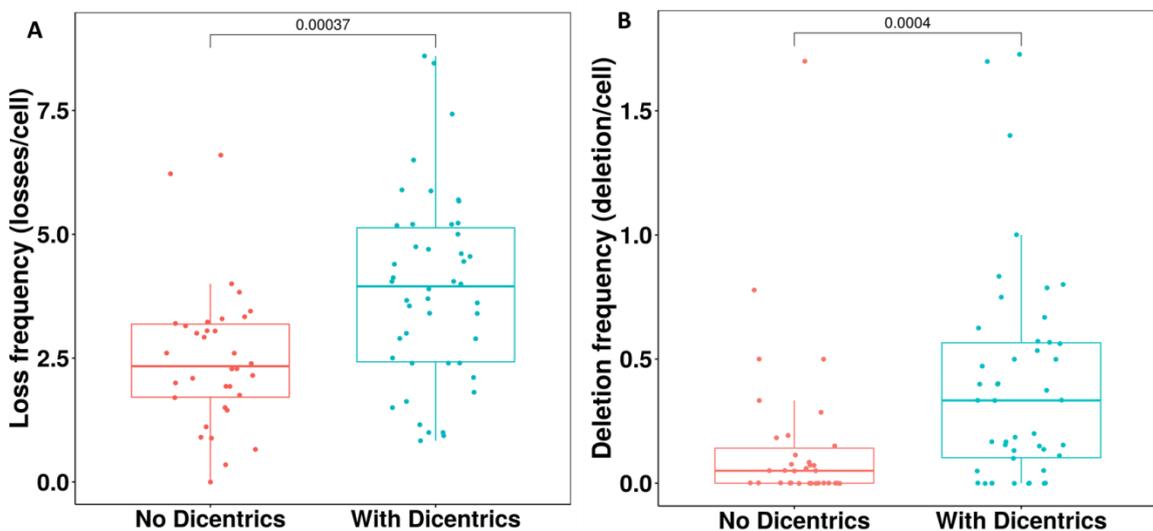
313 Dicentric chromosomes are considered to be an important biomarker of chromosomal instability  
 314 and are associated with complex karyotypes and poor clinical outcomes [31]. Therefore, the  
 315 identification of dicentric chromosomes in patients with cancer or genetic disorders may facilitate  
 316 identification of the disease and thus support the decision for an optimal therapeutic strategy. Dicentric  
 317 chromosomes were found in 28/50 hematological cancer patients and 20/44 patients with genetic  
 318 disorders using TC+M-FISH versus 7/50 and 12/44 by conventional cytogenetics, respectively. A specific  
 319 dicentric chromosome configuration, in which the two centromeres are close to each other, was found in  
 320 70% of cases of cancer patients and those with genetic disorders. Such dicentric chromosomes are easily  
 321 missed by conventional cytogenetics as well as molecular approaches and can be mistaken for  
 322 translocations (Figure 3). In addition, the detection of dicentric chromosomes was related to the presence  
 323 of a complex karyotype (Figure 6).



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Figure 6. The presence of dicentric chromosome is associated with telomere dysfunction and complex karyotype. Sequential analysis shows the presence of clonal dicentric chromosomes and centric rings in this mantle-cell lymphoma patient. These configurations are related to the presence of chromosomal aberrations related to breakage-fusion-bridge cycles, such as  $der(18)t(18,11;5)$  with an interstitial telomere of chromosome 18 and  $der(22)t(22;3;17;20;11;3;11)$ .

There was a significant correlation between the presence of dicentric chromosomes and telomere loss ( $p < 10^{-4}$ ) (Figure 7A) and telomere deletion ( $p < 10^{-4}$ ) (Figure 7B). This underscores the importance of impaired telomere integrity in the initiation and progression of chromosomal instability. In contrast, no significant correlation was observed between telomere length and the presence of dicentric chromosomes.



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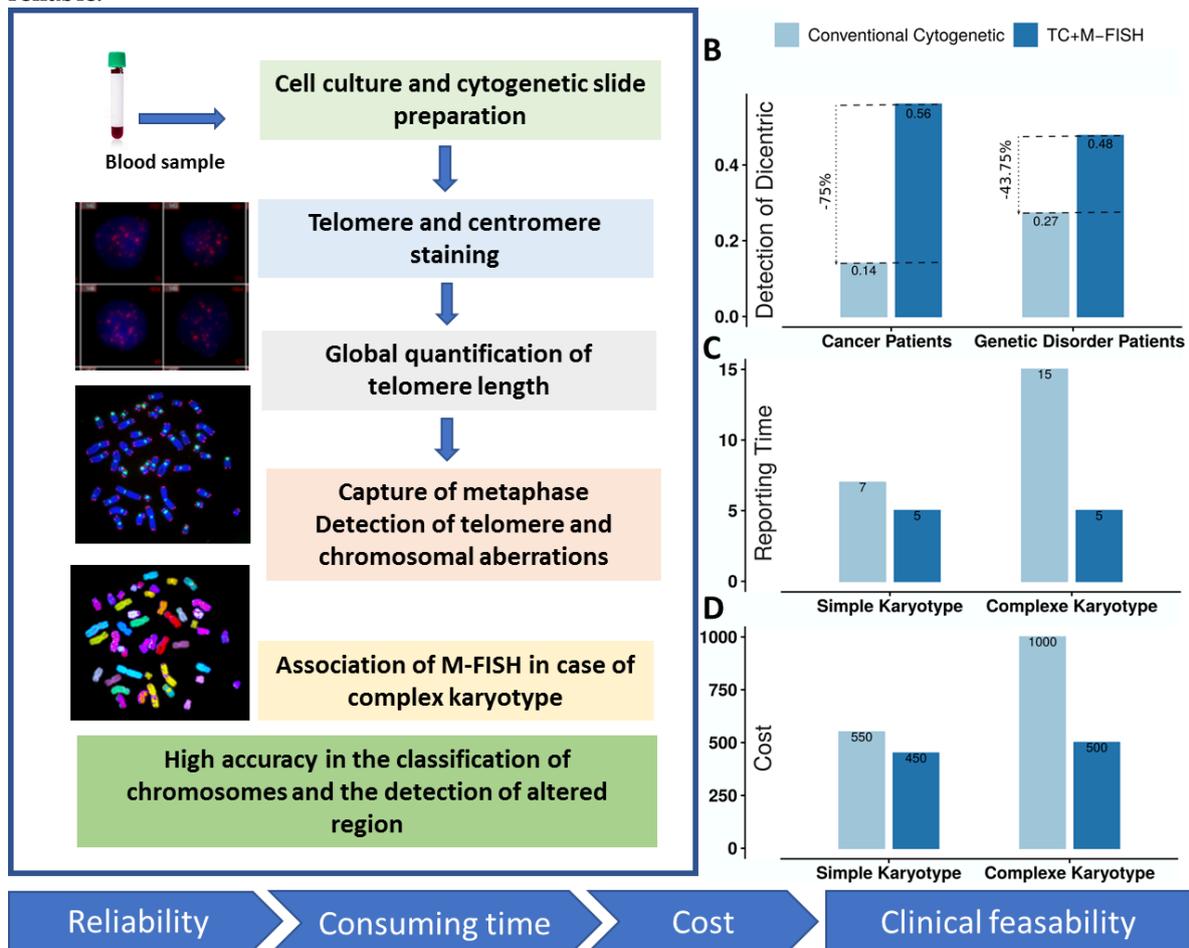
Figure 7. Telomere dysfunction and dicentric chromosome formation. (A) High rate of telomere loss and (B) deletion in patients with dicentric chromosomes compared to those without.

338 3.4. Proof of concept in the clinics

339 Reproducibility, feasibility, and low cost are critical for any new technique destined for routine  
 340 clinical use. We provide data on the evaluation of the time, concordance of results, and cost of testing  
 341 cancer patients and those with genetic disorders using conventional cytogenetics and the  
 342 TC+M-FISH approach for chromosomal analysis (Figure 8).

343 The use of PNA probes allows shorter hybridization times and provides higher signal intensity  
 344 and lower costs than DNA probes for TC staining. Consequently, the introduction of TC staining  
 345 among the techniques commonly used in clinical practice should not increase the costs relative to  
 346 those of conventional cytogenetic analysis. Furthermore, TC staining will dramatically shorten the  
 347 time devoted to the analysis of the results. In addition, TC-M-FISH is a reliable and sensitive  
 348 approach for the analysis of a large number of cells and the detection of clonal expansion and  
 349 chromosomal instability (Figure 8B-D).

350 The assessment of chromosomal aberrations by TC+M-FISH indeed incurs certain additional  
 351 costs required for consumables. However, the technique offers higher specificity and reliability of  
 352 the results, as well as a significant reduction in the time required for analysis relative to that of  
 353 conventional cytogenetics in cases of simple or complex karyotypes. Moreover, the quantification of  
 354 telomere length and telomere aberrations is not possible by conventional cytogenetics. The  
 355 application of an automated approach (TeloScore and ChromoScore) renders the global  
 356 quantification of telomere length, as well as the scoring of telomere aberrations, easier and more  
 357 reliable.



358 Figure 8. Overview of TC+M-FISH for the detection of chromosomal aberrations (A) Description of the TC+M-FISH  
 359 approach. (B) The sensitivity of TC+M-FISH in the detection of dicentric chromosomes in cancer patients and those with  
 360 genetic disorders compared to conventional cytogenetics. (C) Reporting time from the analysis of blood samples, with or  
 361 without complex karyotype, using the TC+M-FISH approach compared to conventional cytogenetics (D)The cost (in euros) of  
 362 the two approaches for the analysis of a simple and complex karyotype, based on the European situation.  
 363

#### 364 4. Discussion

365 Chromosomal instability is known to interfere with treatment responses in cancer patients and  
366 more generally with clinical outcomes in populations exposed to genotoxic agents [32]. However,  
367 until now, the analysis of chromosomal instability has not been incorporated into clinical practice  
368 [33]. The lack of standardization and the uncertainties in terms of optimal cut-offs may account for  
369 its suboptimal utilization and the consequent absence of proof in its clinical usefulness.

370 In particular, the analyses of telomere and centromere sequences have been excluded from  
371 cytogenetic studies in clinical practice. However, telomere and centromere staining has contributed  
372 to improving our knowledge of their role in carcinogenesis, tumor progression, and genetic  
373 disorders.

374 Here, we developed an adaptation of chromosome banding and fluorescence in situ  
375 hybridization to assess telomere dysfunction and chromosomal instability in cytogenetic clinical  
376 practice, not only to identify cancer patients with a high degree of chromosomal instability and  
377 improve their therapy, but also to assess the level of cancer risk in patients with a genetic disorder or  
378 in a population exposed to genotoxic agents. This allowed the detection of aberrations involving  
379 unique and repeated sequences.

380 First, we examined telomeres and sub-telomere regions that play a key role in genome stability  
381 and integrity [34]. Telomere length is a biomarker of cancers and aging disease [20, 35].

382 Although long telomeres may be associated with higher telomerase activity and with cancer  
383 [20, 36, 37], telomere shortening represents the main mechanism of senescence and tumor initiation  
384 and progression. In addition, growing interest in the implication of telomere dysfunction in genetic  
385 disorders has been recently addressed [38, 39]. The development of a faster and more efficient  
386 diagnostic approach for the detection of telomere dysfunction in cytogenetic clinical practice is  
387 necessary to not only better target cancer cells and genetic disorders but also to monitor populations  
388 exposed to genotoxic agents. Using cytogenetic slides, we demonstrated that our approach, based on  
389 the Q-FISH technique, can easily assess mean telomere length and the inter-cellular and the  
390 inter-chromosome heterogeneity of telomere signals. In addition, it was possible to assess telomere  
391 loss, telomere deletion, and the formation of telomere doublets, which are the consequence of  
392 telomere dysfunction. This approach provides multiple advantages for the quantification of  
393 telomere length and telomere aberrations. The visualization of telomere signals in each cell and on  
394 metaphase chromosomes allows the detection of those cells that present very short telomeres and  
395 chromosome uncapping, which play a major role in the senescence and initiation of diseases [40]. In  
396 contrast to most existing high-throughput techniques for telomere quantification, such as  
397 quantitative PCR (qPCR) [41] or Flow-FISH [42], the heterogeneity of telomere length and telomere  
398 aberrations can be scored at the single-cell level and detected on the chromosomes using our  
399 approach. Indeed, we show that the mean telomere length from a defined sample is not necessarily  
400 the best biomarker for telomere dysfunction. Our data contribute to the validation of the concept  
401 that the proportion of cells with short telomeres is a better biomarker for aging and diseases [43].  
402 Surprisingly, telomere loss and deletion, which are age independent in healthy populations, may be  
403 more relevant than mean telomere length or the frequency of cells with short telomeres. Their  
404 frequencies were significantly higher in cancer patients, as well as patients with genetic disorders.  
405 Therefore, these telomere aberrations should be used as more accurate biomarkers for disease risk  
406 stratification.

407 Next, we analyzed the critical role of centromeres in maintaining genome integrity. Increased  
408 evidence has accumulated that centromeric and pericentromeric regions display heterogeneous  
409 alterations in several diseases [22]. Conventional and genomic approaches have demonstrated their  
410 limited capability to assess aberrations related to these regions [18]. The implication of centromeric  
411 and pericentromeric regions in chromosomal aberrations and chromosomal instability is still  
412 unresolved. We demonstrated previously that the application of TC+M-FISH improved the  
413 detection of chromosomal aberrations and their persistence in subsequent cell generations after  
414 exposure to genotoxic agents, such as irradiation [26]. In addition, we demonstrated that the  
415 transmission of dicentric chromosomes was more efficient when both centromeres were very close

416 and centromeres were near telomere sequences. Also, small centric rings demonstrated a higher rate  
417 of stability. These configurations cannot be detected by conventional staining or molecular  
418 cytogenetic approaches. In light of these data, it is necessary to re-evaluate the presence of dicentric  
419 chromosomes and centric rings in cancer patients and those with genetic disorders by TC+ M-FISH.  
420 The presence of dicentric chromosomes in cancer patients and those with genetic disorders has been  
421 related to chromosomal instability, a complex karyotype, and poor clinical outcomes [44, 45]. In a  
422 cohort of 50 cancer patients and 44 patients with genetic disorders, we re-evaluated the presence of  
423 dicentric chromosomes, centric rings, and other chromosomal aberrations to establish a reliable and  
424 robust karyotype and detect the heterogeneity or the mosaics that are known to participate in the  
425 early step of tumorigenesis and the initiation of chromosomal aberrations. Most dicentric  
426 chromosomes detected were characterized by a specific configuration, consisting of the two  
427 centromeres very in close proximity in both populations of patients. These dicentric chromosomes  
428 were not detected using conventional cytogenetic approaches (chromosome banding or FISH).  
429 These results validate our previous data concerning the transmission of chromosomal aberrations  
430 and highlight the implication of centromeric or peri-centromeric regions in the formation of a  
431 specific configuration of dicentric chromosomes [26]. Application of the centromeric FISH technique  
432 has previously been reported for the identification of dicentric chromosomes [46-50].  
433 Multi-centromeric FISH was proposed as a reliable and robust routine technique for the detection of  
434 dicentric chromosomes [50]. Dicentric chromosomes were detected in 51% of analyzed patients with  
435 acute myeloid leukemia. It was reported that these dicentric chromosomes were characterized by  
436 their short intercentromeric distance[47]. Our study confirms these data and provides an attractive  
437 approach to cytogenetic clinical practice with a short time of hybridization, robust signals, and a low  
438 cost relative to multi-centromeric FISH probes.

439 We detected the relationship between telomere dysfunction (telomere loss and deletion) and  
440 chromosomal instability (the presence of dicentric chromosomes) in cancer patients and those with  
441 genetic disorders. These telomere aberrations appear to be shared between genetic disorders and  
442 cancer, with a higher frequency in cancer patients, possibly related not only to the age of these  
443 patients but also to the genetic detriment of cancer [51]. Nevertheless, patients with a genetic  
444 disorder exhibit a higher risk of developing cancer [52, 53]. In addition, we confirm the prominent  
445 role of telomere dysfunction in the formation of the configurations of dicentric chromosomes and  
446 chromosomal instability. In the future, it will be important to elucidate the relationship between  
447 telomere dysfunction and the breakpoints in the centromeric or pericentromeric regions.

448 Current genomics techniques have demonstrated their limitations in the detection of  
449 chromosomal aberrations involving repetitive sequences, such as telomeres and centromeres.  
450 Implementation of our approach as an adjunct to the detection of chromosomal instability makes it  
451 possible to improve the automatic detection of chromosomal aberrations at a high sample  
452 throughput for routine clinic processes and follow-up of populations exposed to genotoxic agents.  
453 Chromosome banding and FISH of telomeres and centromeres provide more accurate and  
454 time-saving detection of these chromosomal aberrations. This technique also paves the way for more  
455 efficient guided genomic studies, including NGS investigations. In addition, these data represent a  
456 first step in the establishment of the bridge between the genome and the chromosome [54],  
457 permitting better detection of chromosomal aberrations and introduction of this approach to the  
458 clinic, providing higher efficiency at a low cost.

## 459 5. Conclusions

460 This study demonstrates the potency of the TC+M-FISH technique as a reliable and robust  
461 method for the detection of telomere dysfunction and chromosomal aberrations. Our data encourage  
462 implementation of this technique as a routine method for research as well as the clinical uses. We  
463 suggest that automation of the entire process and the improvement of databases will make it  
464 possible to improve the detection of chromosomal aberrations and chromosomal instability in the  
465 clinic.  
466

467 **6. Patents**

468 The patented process number is FR1858427 and WO63658

469 **Author Contributions:** Conceived and designed the experiments: R.M.; E.J PC performed the experiments:  
470 R.M., C.B., V.M., N.W., M.M, M.A., C.F., W.K., K.S. and, W.N.; analyzed the data: B.C., E.J. and A.D.;  
471 contributed reagents/materials/analysis tools: N.O., A.P., A.D., T.G., G.D., A.T., C.Y., and P.C. and wrote the  
472 paper: P.V., M.F., P.C.; W.H, S.J. and R.M. All authors have read and agreed to the published version of the  
473 manuscript.

474 **Funding:** This research received no external finding

475

476 **Acknowledgments:** We are grateful to the nurses, technologists and secretaries of the different  
477 departments for their support and participation in this study. We are indebted to I. Benabdelaziz for preparing  
478 the manuscript

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

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