

LETTER TO THE JOURNAL

Unraveling the routes to distant metastases in colorectal cancer: Tumor deposits and lymph node metastases as the gateway

Currently, lymph node metastases (LNM) are seen as the gateway to distant metastases in CRC and play a crucial role in the Tumor Node Metastasis (TNM) staging system [1, 2]. Tumor deposits (TD) have been identified as another histological feature with a strong prognostic impact but are currently only deemed clinically relevant in the absence of LNM [2, 3]. TD are clusters of tumor cells in the fat surrounding the bowel and are often associated with nerves, vessels and lymphatic tissue, giving the cancer cells access to multiple routes of spread [4].

To study whether TD could indeed form an alternative gateway to distant metastases, we have used polyG-based phylogenetics on formalin-fixed paraffin-embedded (FFPE) tissue to investigate the role of histologically defined TD and LNM in CRC evolution and distant metastatic spread to the liver and peritoneum.

We collected data from patients who were diagnosed with colorectal liver or peritoneal metastases between January 2015 and January 2022 and not previously treated with chemotherapy. DNA was isolated from the TD and LNM (Figure 1A), normal tissue (which was sampled at the edge of the resection specimen) and distant metastases. Three distinct tissue parts of the primary tumor were sampled to account for intra-tumor heterogeneity (Figure 1B), yielding a total of 480 samples (Supplementary Table S1). Polymerase chain reaction (PCR) was used to genotype up to 24 polyG regions for every sample (Supplementary Table S2). Sequentially, phylogenetic trees were constructed based on the peak distribution of all PCR results from the polyG regions using an analysis pipeline previ-

ously developed by Naxerova et al. in R software (version 4.3.1) [5]. Phylogenetic trees are graphical representations of the evolutionary relationship between biological entities, in this case, the different tissue samples. After quality control of PCR results (i.e., poor quality DNA), impurity (i.e., contamination of samples with non-tumorous cells, Supplementary Figure S1), and branching confidence (i.e., values < 50% were deemed unreliable), 47 CRC patients with a total of 43 individual liver metastases and 30 individual peritoneal metastases were included in the final results (Supplementary Table S3-S5). The distribution of origins (TD, LNM, primary tumor or a mix of these) was compared between the group of liver and the group of peritoneal metastases using Fisher's exact test. Potential confounding clinicopathological factors were analyzed using the Kruskal-Wallis of Fisher's exact test. *P* values < 0.05 were considered significant. Full descriptions of the materials and methods are provided in the Supplementary Materials.

Phylogenetic analysis showed that the primary tumor, TD, and LNM could be possible origins of liver metastases. An exemplary phylogenetic tree is shown in Figure 1C, where different subclones gave rise to different LNMs, and several liver metastases with common ancestry were related to the TD. Taken together, TD, LNM, primary tumor or a mix of these were found to be the origin of liver metastases in 23%, 23%, 23% and 30%, respectively (Figure 1D). Several clinicopathological variables were tested for biasing the results, of which only the number of included TDs was significantly different between the liver metastases related to TD and PT (*P* = 0.03), all other comparisons showed no differences significantly with the number of metastases related to TD (Supplementary Figures S2-S3). Our data show that LNM can form a gateway to liver metastases in 23% of cases, which is in line with an earlier smaller study, where 0-35% of liver metastases were found to be related to LNM [5]. These results are in contrast with the idea that LNM is the main gateway to distant metastases, which is the basis for the current TNM staging system.

List of Abbreviations: CMO, Centrale Commissie Mensgebonden Onderzoek; CRC, Colorectal cancer; DNA, Deoxyribonucleic acid; FFPE, Formalin fixed paraffin embedded; H&E, Hematoxylin & Eosin stained; JSD, Jensen-Shannon distance; LNM, Lymph node metastasis; PALGA, Pathologisch Anatomisch Landelijk Geautomatiseerd Archief; PAMM, Laboratoria voor Pathologie en Medische Microbiologie Nederland; PCR, Polymerase chain reaction; PolyG, Polyguanine; TD, Tumor deposit; TNM, Tumor Node Metastasis.

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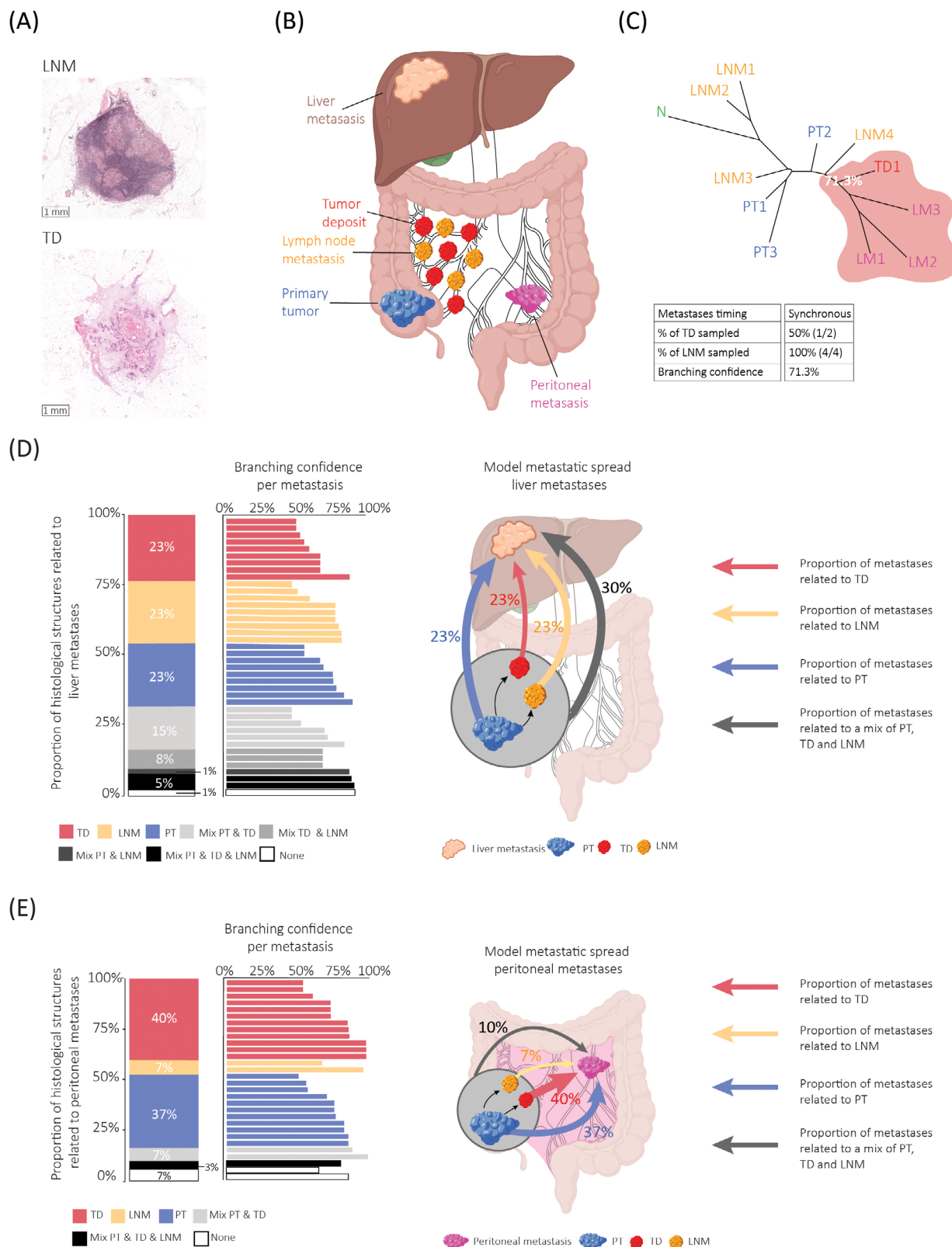


FIGURE 1 The phylogenetic analysis of tumor deposits (TD) and lymph node metastases (LNM) as gateways for distant metastatic spread in colorectal cancer (CRC). (A) Histology of LNM (upper) and TD (lower) on hematoxylin & eosin stained slides. (B) For every included case, DNA was isolated from all TD, all LNM, normal tissue, distant metastases (liver and/or peritoneum). From the primary tumor, DNA was isolated from 3 different areas to account for intratumor heterogeneity. (C) Phylogenetic trees were constructed for all included cases. This phylogenetic tree shows liver metastases that were related to a TD with a branching confidence of 71.3%. (D) Bar graph showing all liver metastases and their potential origin in the form of TD, LNM, primary tumor or a combination of these. Also, there were some cases where no evolutionary relation was found between distant metastases and TD, LNM or primary tumor. For these cases, the origin category is

Importantly, TD were identified as an alternative gateway in 23% of liver metastases, which confirms the prognostic evidence that shows an increased risk of liver metastases in patients with TD [3].

In contrast to liver metastases, peritoneal metastases were most often related to TD with 40% of cases and second most often to the primary tumor with 37%. LNMs or a mix of the different structures formed the origin in 7% and 10% of peritoneal metastases, respectively (Figure 1E). These results provide phylogenetic evidence that supports previous studies showing that the presence of TDs is associated with an increased risk of peritoneal metastases [6].

This study demonstrated a clear difference in the evolution of liver and peritoneal metastases, with a significantly different distribution of origins ($P < 0.05$, Figure 1D-E) and 40% of peritoneal metastases versus 23% of liver metastases being related to TD. This could be explained by biologically different pathways to metastases. Liver metastases are thought to develop through the invasion-metastasis cascade where tumor cells spread to distant organs via the circulatory system [7]. Both LNMs and TDs formed possible gateways to liver metastases in the phylogenetic analyses and have their own way of accessing the systemic circulation. For LNMs, mouse studies showed that tumor cells could either invade small blood vessels surrounding the lymph node or travel through the lymphatic system and enter the systemic circulation at the thoracic duct [8]. Still, the exact mechanism is being widely debated, and in humans, the mechanism remains unclear. For TDs, it is more widely accepted that they have access to the blood circulation as it is reflected in their histology [4]. In line with literature, our study demonstrated a vascular component in 69% of TD related to liver metastases (Supplementary Figure S4). An alternative to the invasion-metastasis cascade has been proposed for peritoneal metastases involving more direct migration of tumor cells to the peritoneum [7]. We previously showed that the molecular phenotype needed for tumor cells to actively migrate to and invade the peritoneum is overexpressed in TD compared with LNM [9]. Therefore, it is likely that tumor cells with the ability to form TD are also capable of further migration through the serosal surface and invasion of the peritoneum, more so than the tumor cells in LNM.

There are some limitations to this study. Firstly, although all cases complied with the guideline quality of more than 12 sampled lymph nodes per CRC, and TD were scored by two pathologists and an expert researcher, there is a small chance that some TD or LNM were missed during the sampling of resection specimens. Secondly, it would be recommended that all phylogenetic studies be validated using a different method, such as whole genome or exome sequencing, to show whether the results are generalizable and robust [10]. Thirdly, due to time and financial restraints, this study only included the most common metastatic sites in CRC, liver and peritoneum. Including other metastatic sites would provide a more comprehensive overview of the process of metastatic spread in CRC.

In conclusion, with the use of phylogenetic analysis, this study shows that both TDs and LNMs can be related to liver or peritoneal metastases in CRC. Moreover, the origin of metastases differs per metastatic site, with liver metastases being equally related to TDs and LNMs and peritoneal metastases being significantly more often related to TDs. Therefore, future research should focus on whether patients with TDs are at increased risk of peritoneal metastases, as this could impact follow-up and treatment decisions. Also, since we showed that a significant number of liver and peritoneal metastases were related to TDs, it should be questioned whether the current method of including TDs as less important than LNMs in the staging for CRC patients is justified. We propose that TDs should merit more attention by including them as equally important to LNMs in the nodal category and continuing to register them for future research.

AUTHOR CONTRIBUTIONS

Conceptualization: Gina Brown, Femke Simmer, Iris Dionne Nagtegaal. Methodology: Nelleke Pietronella Maria Brouwer, Ayse Selcen Oguz Erdogan, Niek Hugen, Femke Simmer, Iris Dionne Nagtegaal. Funding acquisition: Gina Brown, Iris Dionne Nagtegaal. Acquisition and analysis of data: Nelleke Pietronella Maria Brouwer, Ayse Selcen Oguz Erdogan, Shannon van Vliet, Natasja Rutgers. Statistical analysis: Nelleke Pietronella Maria Brouwer, Ayse Selcen Oguz Erdogan, Shannon van Vliet,

called 'none'. The branching confidence is provided to show the confidence of every branch and does not show major differences between the different origin groups. The results are summarized in an anatomical sketch (shown on the right). (E) Bar graph showing all peritoneal metastases and their potential origin in the form of TD, LNM, primary tumor or a combination of these. Also, there were some cases where no evolutionary relation was found between distant metastases and TD, LNM or primary tumor. For these cases, the origin category is called 'none'. The branching confidence is provided to show the confidence of every branch and does not show major differences between the different origin groups. The results are summarized in an anatomical sketch (shown on the right). The Fisher's exact test was used to compare the distribution of the origins of liver and peritoneal metastases and showed a significant difference in the distributions ($P < 0.05$). Abbreviations: tumor deposits, TD; lymph node metastases, LNM; colorectal cancer, CRC; deoxyribonucleic acid, DNA; primary tumor, PT; normal tissue, N; liver metastasis, LM.

Natasja Rutgers, Femke Simmer. Material support: Nikki Knijn, Gesina van Lijnschoten, Jessica Juliana Tan, Gina Brown. Writing – original draft: Nelleke Pietronella Maria Brouwer, Ayse Selcen Oguz Erdogan, Niek Hugen, Femke Simmer, Iris Dionne Nagtegaal. Writing – review & editing: Nelleke Pietronella Maria Brouwer, Ayse Selcen Oguz Erdogan, Shannon van Vliet, Natasja Rutgers, Nikki Knijn, Gesina van Lijnschoten, Jessica Juliana Tan, Johannes Hendrik Willem de Wilt, Niek Hugen, Gina Brown, Femke Simmer, Iris Dionne Nagtegaal.

ACKNOWLEDGMENTS

None to be declared.

CONFLICT OF INTEREST STATEMENT

All authors declare no conflicts of interest.

FUNDING INFORMATION

This study was supported by the Dutch Cancer Society (KUN 2019-12640).

DATA AVAILABILITY STATEMENT

Raw data on peak patterns from all polyguanine regions obtained by PCR per patient are provided on the data repository Zenodo and are publicly available via Zenodo <https://doi.org/10.5281/zenodo.11119676>. All the other data are available upon reasonable request from the corresponding author.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was performed according to the Dutch “Federa, Human Tissue and Medical Research: Code of Conduct for Responsible Use (2011)” regulations, not requiring patient informed consent. This study was approved by the Ethics Committee (CMO) of the Radboudumc (approval number 2020-7172).

Nelleke Pietronella Maria Brouwer¹ 
 Ayse Selcen Oguz Erdogan¹
 Shannon van Vliet¹
 Natasja Rutgers¹
 Nikki Knijn²
 Gesina van Lijnschoten³
 Jessica Juliana Tan⁴
 Johannes Hendrik Willem de Wilt⁵
 Niek Hugen^{6,7}
 Gina Brown⁸
 Femke Simmer¹
 Iris Dionne Nagtegaal¹

¹Department of Pathology, Radboud University Medical Center, Nijmegen, Gelderland, The Netherlands

²Department of Pathology, PathologyDNA, Arnhem, Gelderland, The Netherlands

³Department of Pathology, Eurofins/Laboratoria voor Pathologie en Medische Microbiologie Nederland, Eindhoven, Noord-Brabant, The Netherlands

⁴GI Cancer Imaging Research Unit, The Royal Marsden Hospital, Sutton, London, United Kingdom

⁵Department of Surgery, Radboud University Medical Center, Nijmegen, Gelderland, The Netherlands

⁶Department of Surgery, Netherlands Cancer Institute, Amsterdam, Noord-Holland, The Netherlands

⁷Department of Surgery, Rijnstate Hospital, Arnhem, Gelderland, The Netherlands

⁸Imperial College London Hammersmith Campus, London, London, United Kingdom


Correspondence

Nelleke Pietronella Maria Brouwer, Department of Pathology, Radboud University Medical Center, Nijmegen, The Netherlands.

Email: Nelleke.brouwer@radboudumc.nl

Nelleke Pietronella Maria Brouwer is responsible for all aspects of this work, including descriptions, illustrations and data accuracy.

ORCID

Nelleke Pietronella Maria Brouwer  <https://orcid.org/0000-0002-2268-9668>

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.