UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

Detection of circulating tumor cells in renal cancer — the impact of no-touch surgery

Tito Miguel Palmela Leitão

Orientadores: Professor Doutor Luís António Marques da Costa
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Tese especialmente elaborada para obtenção do grau de Doutor em **Medicina**
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2024

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As opiniões expressas nesta publicação são da
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"We are what we repeatedly do. Excellence, therefore, is not an act, but a habit."

Aristotle

PREFÁCIO

Afirmar que um cirurgião tem umas mãos de ouro será comparável a dizer que um icónico e potente carro desportivo tem uns pneus de ouro. A arte não está no pincel ou cinzel que lhe dá forma. Tal como na arte, também o acto cirúrgico é 90% cerebral, sempre com um forte coração como suporte. É um processo intelectual complexo que é aplicado pelas mãos do cirurgião com gestos singularmente simples. É por isso que a excelência em cirurgia não é mais que uma busca constante pelo aperfeiçoamento do gesto cirúrgico, reconhecendo humildemente todas as limitações técnicas, gnósicas e também humanas, de quem procura cuidar da pessoa doente, de lhe aliviar o sofrimento. É um movimento intelectual contínuo. Foi esta consciência, esta missão, que motivou e norteou este projeto de doutoramento.

Tive a enorme felicidade de receber essa centelha perene, como um testemunho, dos meus mestres cirúrgicos, o Dr. João Carvalho Varela (que descanse em paz eterna), o Dr Tomé Matos Lopes, o Dr Eric Mandron e o Dr Henrique Vasconcelos Dias. A eles devo tudo na Urologia e muito na vida. Foi com eles que aprendi a Ser Médico. A Ser Cirurgião. Foi deles que recebi a dádiva de poder tocar o corpo e a vida de tantas pessoas doentes, com a intenção de curar ou, pelo menos, paliar. Se isso não é mágico, nem que seja como simples conceito filosófico, não saberei então o que o será. Espero que este trabalho possa acrescentar uma pequena partícula a essa centelha. E espero que a possa passar ao próximo, para que nunca se extinga.

O **Capítulo I** consiste numa introdução geral abordando os tópicos abordados na Tese, baseada na revisão da literatura. No **Capítulo II** são explanados os objetivos do projecto. Os **Capítulos III a V** contém os três artigos de investigação publicados em revistas internacionais de elevado factor de impacto, do primeiro quartil na respectiva área do conhecimento, e que reportam as diversas fases do trabalho experimental realizado ao longo deste projecto. Finalmente, o **Capítulo VI** consiste numa discussão integradora de todo o projecto e em perspectivas futuras.

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Ao longo deste projecto de investigação no contexto do Programa Doutoral do CAML, tive a felicidade de trabalhar com pessoas extraordinárias, dos pontos de vista profissional, académico e pessoal. Esta tese de doutoramento é o resultado do longo e intenso trabalho de uma vasta equipa, com a qual tive o privilégio de trabalhar. Por essa razão, deixo os meus sinceros agradecimentos:

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Aos meus pais, a quem devo tudo, agradeço profundamente todo o amor, educação, tudo o que sempre fizeram por mim e o orgulho que sentem pelo meu trajecto profissional.

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RESUMO

O carcinoma de células renais (CCR) representa 430.000 casos por ano diagnosticados em todo o mundo e mais de 140.000 mortes. O diagnóstico e o acompanhamento do CCR ainda é baseado na identificação de massas tumorais em exames de imagem. O limiar para identificar uma massa renal ou metástase é de aproximadamente 1 cm, o que implica, muito possivelmente, que só podemos detectar a doença quando mais de 100.000.000 de células malignas se desenvolveram. Atualmente, não há nenhum biomarcador capaz de detectar a doença localizada precocemente, recidivas precoces ou micrometastização antes que massas visíveis sejam identificadas por exames de imagem. Para melhorar a sobrevivência dos pacientes com CCR, é fundamental identificar um biomarcador clinicamente útil.

O potencial das células tumorais circulantes (CTCs) tem sido explorado como biomarcador em vários tipos de tumores, como mama, cólon e próstata. Diversos estudos encontraram uma correlação entre a presença e/ou contagens de CTCs e medidas de prognóstico nestes tumores, bem como no CCR. As CTCs têm o potencial de vir a ser um biomarcador importante para o CCR. No entanto, a evidência disponível é limitada e os métodos de detecção são heterogéneos e sem padronização, limitando a sua aplicação clínica.

Realizamos, assim, uma revisão sistemática da literatura sobre técnicas de enriquecimento e detecção de CTCs, com o objectivo de estruturar a base de conhecimento atual e ajudar a guiar a investigação futura, para além de procurar determinar o seu papel atual como biomarcador no CCR.

A triagem dos artigos identificou 54 estudos, que mostraram uma ampla heterogeneidade, baixo nível de evidência e alto risco de viés. Várias plataformas de detecção de CTCs e marcadores moleculares foram utilizados, mas nenhuma se mostrou superior. Os resultados foram organizados em três tópicos: técnicas de enriquecimento de CTCs, métodos de detecção de CTCs e desempenho das CTCs como biomarcador diagnóstico, prognóstico e de resposta ao tratamento.

Diversos métodos de enriquecimento de CTCs têm sido usados, podendo ser organizados em quatro categorias: técnicas baseadas em anticorpos, em densidade, em tamanho e em eletroforese. Após o enriquecimento da amostra, a detecção das CTCs é realizada por uma de cinco técnicas: imunocitoquímica, reação em cadeia da polimerase com transcriptase reversa (RT-PCR), critérios citomorfológicos, imunofluorescência por citometria de fluxo e hibridização *in situ* por fluorescência (FISH). Plataformas baseadas exclusivamente em marcadores epiteliais são inadequadas no CCR devido à frequente transição epitelial-mesenquimal (EMT) que as CTCs sofrem.

A detecção e contagem de CTCs parecem ter correlação com o estadiamento e a sobrevida, embora a evidência seja inconsistente. Com esta revisão sistemática, concluímos que a pesquisa sobre CTCs ainda está numa fase exploratória, especialmente no CCR, e que são necessários mais estudos para padronizar as técnicas, os marcadores moleculares, e as próprias definições das CTCs.

Um estudo comparou diferentes plataformas de isolamento e detecção de CTCs no CCR e concluiu que os sistemas baseados em tamanho celular são os mais eficientes.

Portanto, selecionamos e definimos com objetivo testar e validar clinicamente o RUBYchip™, uma nova plataforma de detecção de CTCs por microfluídica baseada em tamanho celular, em doentes de CCR.
Este dispositivo captura CTCs ao passar a amostra de sangue por uma série de capilares interconectados que alimentam várias câmaras de filtragem de células com fileiras transversais de micropilares, que permitem que os leucócitos, mais deformáveis, a atravessem suavemente, retendo células maiores e com maior rigidez, como as CTCs. Estas são posteriormente identificadas através de marcação com anticorpos para CK (marcador epitelial), vimentina (marcador mesenquimal, para detectar CTCs mesenquimais ou em transição epitélio-mesenquima), DAPI e CD45 (controle negativo para leucócitos).

A eficácia média de captura de CTCs do dispositivo foi de 74,9% em experiências de *spiking,* usando três linhas celulares de CCR diferentes. A validação clínica foi

realizada num grupo de 18 doentes, com uma taxa média de detecção de 77,8%. A contagem média total de CTCs foi de 6,4 (0-27), 101,8 (0-255) e 3,2 (0-10), e a média de CTCs mesenquimais (isoladas ou em aglomerados) foi de 0,0, 97,6 (0-255) e 0,2 (0-1), para os grupos de doentes com doença localizada (M0), com doença metastática (M1) sem tratamento e M1 em progressão sob tratamento, respectivamente. Aglomerados de CTCs foram detectados em 25% e 60% nos pacientes M0 e M1 sem tratamento, respectivamente. Em conclusão, os resultados demonstraram que o RUBYchip™ é uma plataforma eficaz e fidedigna para detecção de CTCs no CCR.

A cirurgia é o tratamento com intenção curativa de eleição no CCR. Os cirurgiões oncológicos buscam continuamente o desenvolvimento de novas técnicas para melhorar os resultados cirúrgicos e a sobrevida dos seus doentes. No entanto, a taxa de recidiva ou metastização é ainda de cerca de 20 a 40% em doentes com tumores localizados.

Até ao momento, apenas um pequeno número de estudos exploratórios sobre CTCs no contexto perioperatório no CCR foram realizados. A maioria revela um aumento das contagens de CTCs no pós-operatório imediato e uma diminuição nos dias e meses subsequentes.

A procura da melhoria dos resultados cirúrgicos oncológicos, levou à criação do conceito de ressecção tumoral "sem toque", introduzido pela primeira vez em 1977 no cancro colorectal. Desde então, estes tipos de técnicas foram estudadas em diversos tipos de cancro, na maioria dos casos revelando uma diminuição nas taxas de detecção de CTCs em relação às técnicas convencionais. Tanto quanto sabemos, este tipo de técnicas não foram ainda estudadas no CCR.

Portanto, desenhamos um ensaio clínico prospectivo aleatorizado para avaliar se uma nefrectomia radical laparoscópica (NRL) "sem toque" (ST) reduziria a liberação de CTCs em comparação com uma NRL convencional (C). Amostras de sangue foram colhidas na chegada ao bloco operatório (S0), após a extração da peça operatória (S1), no primeiro dia de pós-operatório (D1) e ao 30º dia (D30).

As CTCs foram analisadas com o RUBYchip™, que havíamos validado previamente.

De setembro de 2021 a abril de 2022, 34 pacientes foram incluídos e aleatorizados. Não foi encontrada diferença significativa nas variações de CTCs e CMCs entre o grupo da NRL ST e C. As taxas de complicações foram semelhantes.

As taxas de detecção de CTCs foram de 0% e 6,25% em S0 e S1, 18,75% e 12,5% em D1, e 6,25% e 12,5% em D30, respectivamente para os grupos ST e T. As taxas de detecção de CMCs foram de 68,9% e 75,0% em S0, 50,0% e 81,25% em S1, 37,5% e 56,25% em D1 e 50,0% e 43,75% em D30, respectivamente para os grupos ST e C.

Foi observada uma diminuição progressiva de CMCs após a cirurgia no grupo C, principalmente no D1, de 4,78 para 1,64 CMCs/7,5mL de sangue (p=0,035).

A técnica ST para a NRL não mostrou uma redução significativa na libertação de CTCs ou CMCs ou uma melhoria da sobrevida livre de progressão ou global em comparação com a técnica convencional. No entanto, a nefrectomia ST foi mais rápida e tão segura quanto a técnica convencional, o que, só por si, confere uma vantagem.

Neste estudo, o grupo de controlos saudáveis não apresentou células circulantes. No entanto, foram encontradas contagens elevadas de CMCs no grupo de controlo de doentes com inflamação crónica e em doentes com oncocitomas. Estas contagens foram semelhantes às dos doentes com CCR (p=0,460). Este foi um achado surpreendente, uma vez que na literatura até ao momento não foram encontradas CTCs ou CMCs com os critérios usados neste estudo. No entanto, tanto quando conhecemos, nenhum estudo incluiu um grupo de controlo com inflamação crónica.

Este achado levanta a questão de qual a origem destas CMCs. A comunidade científica tem considerado estas células como CTCs mesenquimais. Portanto, o

presente estudo levanta a questão de saber se essas células são de facto CTCs ou se têm outras origens, como por exemplo serem um subtipo de leucócitos sem marcação para CD45 ou fibroblastos associados ao cancro.

A libertação e cinética de CTCs perioperatórias ainda foram pouco estudadas e o seu impacto clínico não foi ainda esclarecido. Este estudo demonstrou que não parece haver vantagem na laqueação precoce do pedículo renal em termos de libertação de células ou de sobrevida. Será, então, que o último dos princípios de Robson caiu? Estudos com amostras de tamanho adequado são necessários para corroborar esta afirmação. Adicionalmente, uma análise mais detalhada das CTCs e CMCs, bem como a sua interação com o sistema imunológico, podem melhorar a nossa compreensão do CCR e ajudar a optimizar os protocolos de tratamento desta doença.

Palavras-chave: células tumorais circulantes, cancro do rim, biópsia líquida, cirurgia *no-touch*, nefrectomia radical.

ABSTRACT

Renal cell carcinoma (RCC) accounts for 430,000 cases diagnosed worldwide each year and more than 140,000 deaths. RCC diagnosis and follow-up are still based on the identification of tumor masses on cross-sectional imaging. The threshold for identifying a renal mass or lymph node metastasis is approximately 1 cm, which implies that we can only detect the disease when more than 100,000,000 cancer cells have developed. There is currently no biomarker capable of detecting early localized disease or early relapses before gross masses are visible on imaging. To improve RCC patients' survival, it is paramount to identify this biomarker.

The potential of circulating tumor cells (CTCs) has been explored as a biomarker in various types of tumors, such as breast, colon, and prostate. Several studies have found a correlation between CTC presence and/or counts and prognostic outcome measures in these tumors, and also in RCC. CTCs have a potential role as one of the missing RCC biomarkers. However, the available evidence is limited, and detection methods are heterogeneous and lack standardization, hindering its clinical use.

Hence, we performed a systematic review of the literature on CTC enrichment and detection methods, to structure the present knowledge base and guide future research and determine the current role of CTCs as a biomarker in RCC.

Full-text screening identified 54 studies, which showed a wide heterogeneity, low evidence level, and a high risk of bias. Various CTC detection platforms and molecular markers have been used, but none has proven to be superior.

Retrieved evidence was organized into three topics: CTC enrichment techniques, CTC detection methods, and CTC performance as a diagnostic, prognostic, and treatment response biomarker.

A multitude of enrichment methods have been used to date, which can be organized in four different categories: antibody-, density-, size-, and

electrophoresis-based methods. After sample enrichment, CTC detection is performed by one of five different techniques: immunocytochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), cytomorphological criteria, flow cytometry immunofluorescence, and fluorescence in situ hybridization (FISH).

CTC detection and CTC count seem to correlate with staging and survival outcomes, although evidence is inconsistent. We concluded that CTC research is still in an exploratory phase, particularly in RCC, and that further studies are still necessary to achieve a standardization of techniques, molecular markers, CTC definitions, and terminology.

Platforms relying solely on epithelial markers are inappropriate in RCC due to the frequent epithelial-to-mesenchymal transition (EMT) that CTCs undergo. A study comparing different isolation platforms for RCC CTC detection has concluded that cell-size-based systems are the most efficient.

Therefore, we aimed to test and clinically validate the RUBYchip™, a new microfluidics label-free CTC detection platform, in RCC patients. CTCs were captured by passing blood samples through a series of interconnected capillaries into several cell-filtering chambers with transverse single rows of micropillars, which allow deformable white blood cells gently to flow through, and retain larger, more rigid cells, like CTCs. These are then identified by staining with antibodies to CK (epithelial marker), vimentin (to detect mesenchymal and epithelial-to-mesenchymal transitioned CTCs), DAPI, and CD45 (negative leukocyte control).

The average capture efficiency was 74.9% in spiking experiments using three different RCC cell lines. Clinical validation was done in a cohort of 18 patients, with an average CTC detection rate of 77.8%. The average total CTC count was 6.4 (0-27), 101.8 (0-255), and 3.2 (0-10), and the average of mesenchymal CTCs (single and in clusters) was zero, 97.6 (0-255), and 0.2 (0-1), for M0, M1 treatment-naive (M1TN), and M1 progressing-under-treatment (M1TP) groups, respectively. CTC clusters were detected in 25% and 60% of M0 and M1TN

patients, respectively. In conclusion, our results demonstrated that RUBYchip™ is an effective and reliable CTC detection platform in RCC.

Surgery is the best treatment with curative intent for RCC, although local recurrence or distant metastasis can occur in 20-40% of patients treated for localized tumors. Oncologic surgeons keep developing new techniques to improve surgical outcomes for their patients.

A few exploratory studies on perioperative CTCs have been conducted in the RCC setting so far. Most reveal an increase in counts at D1 and a decrease in the subsequent days.

The concept of no-touch tumor resection was first introduced in 1977 in colorectal cancer. Since then, it has been studied in several cancer types with most revealing a decrease in CTC detection rates, but to our knowledge, not in RCC.

Hence we designed a randomized controlled trial to determine if a no-touch (NT) laparoscopic radical nephrectomy (RN) would reduce CTC and circulating mesenchymal cell (CMC) release compared to a conventional RN. Blood samples were collected at OR arrival (S0), specimen extraction (S1), D1, and D30. CTCs were analyzed with the RUBYchip™.

From September 2021 to April 2022, 34 patients were randomized. There was no significant difference between intervention groups in CTC and CMC variations between time points. Complication rates were similar.

CTC detection rates (DR) were 0%, and 6.25% at both S0 and S1, 18.75% and 12.5% at D1, and 6.25% and 12.5% for D30, respectively for NT and C groups. CMC DRs were 68.9%, and 75.0% at S0, 50.0%, and 81.25% at S1, 37.5%, and 56.25% at D1, and 50.0%, and 43.75% at D30, respectively for NT, and C groups.

There was a progressive decrease in CMCs after surgery in the C group, mainly at D1, from 4.78 to 1.64 CMCs/7.5mL of blood (p=0.035).

The NT technique for laparoscopic RN did not show a significant reduction in CTC or CMC release or improvement in PFS or OS compared to the C technique. However, NT RN proved to be faster and as safe as the C technique.

In this study, healthy controls showed no circulating cells. However, high CMC counts were found in chronic inflammation controls and oncocytoma patients, similar to RCC patients (p=0.460). This was a surprising finding since control groups in the previous literature showed no CTC or CMC presence with the criteria used in the present study. However, to our knowledge, no study to date has included a chronic inflammation control group. This raises the question of the origin of these CMCs. The scientific community has been naming these cells as mesenchymal CTCs. Hence, the present study raises the question of whether these cells are indeed CTCs or if they have other origins, like a subset of WBC without CD45 or cancer-associated fibroblasts.

Perioperative CTC release and kinetics are still poorly understood and their clinical significance is unclear. However, this study proves no advantage in early pedicle ligation. Has the last of the Robson principles fallen? More properly sized studies are warranted to further support the claim. Furthermore, a more comprehensive analysis of CCs and their interaction with the immune system can improve our understanding of RCC and improve future treatment protocols.

Keywords: circulating tumor cells, kidney cancer, liquid biopsy, no-touch surgery, radical nephrectomy.

ABBREVIATIONS

aPTT	activated partial thromboplastin clotting time
BHD	Birt-Hogg-Dubé
BMI	body mass index
C	conventional
CA9	carbonic anhydrase IX
CAF	cancer-associated fibroblast
CC	circulating cell
cCAF	circulating cancer-associated fibroblast
ccRCC	clear cell RCC
CD45	cluster of differentiation
CK	cytokeratin
CMC	circulating mesenchymal cell
CRP	C-reactive protein
CSS	cancer-specific survival
CT	computerized tomography
CTC	circulating tumor cell
ctDNA	circulating tumor DNA
D1	post-operative day 1
D30	post-operative day 30
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DR	detection rate
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
FDA	food and drug administration
FH	fumarate hydratase
FISH	fluorescence in situ hybridization
FLCN	folluculin
HIF	hypoxia-inducible factor
HLRCC	hereditary leiomyomatosis renal cell carcinoma
HPRC	hereditary papillary renal carcinoma

HR	hazard ratio
INL	iberian institute of nanotechnology
ISUP	international society of urological pathology
KC	kidney cancer
LPN	laparoscopic partial nephrectomy
LRN	laparoscopic radical nephrectomy
LRN	laparoscopic radical nephrectomy
M0	non-metastatic
M1	metastatic
M1TN	metastatic patient treatment naive
M1TP	metastatic patient progressing-under-treatment
MACS	magnetic-activated cell sorting
MAPK/ERK	mitogen-activated protein kinase/extracellular signal-regulated kinase
MET	mesenchymal-to-epithelial
MHC	major histocompatibility complex
miRNA	micro RNA
mRCC	metastatic RCC
MRI	magnetic resonance imaging
NK	natural killer
NLR	neutrophil-to-lymphocyte
NT	no-touch
OPN	open partial nephrectomy
ORN	open radical nephrectomy
OS	overall survival
p	p-value
PFS	progression-free survival
PN	partial nephrectomy
PT	prothrombin time
RBC	red blood cell
RCC	renal cell carcinoma
RCT	randomized controlled trial

RN	radical nephrectomy
RNA	ribonucleic acid
RR	relative risk
RT-PCR	reverse transcriptase-polymerase chain reaction
TAN	tumor-associated neutrophil
TGF	transforming growth factor
TKI	tyrosine kinase inhibitors
TNM	tumor node metastasis
VHL	Von Hippel-Lindau
Vim	vimentin
WBC	white blood cell
WHO	world health organization

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FIGURES

1. OVERALL INTRODUCTION

1.1. RENAL CELL CARCINOMA

1.1.1. Epidemiology

Kidney Cancer (KC) is the 14th most common malignancy, with a global incidence of 431,288 cases in 2020, of which 138,611 were in Europe [1,2]. Incidence is considerably higher in Europe and North America compared with other regions, ranging from 2.09 cases per 100,000 inhabitants (age-standardized rate) in Central Africa to 24.7 in North America[2]. Renal cell carcinoma (RCC) accounts for the great majority (90%) of KC cases and comprises different subtypes, each originating from specific cells within the kidney[2]. The peak incidence of RCC occurs between 60 and 70 years of age, with a 3:2 ratio of men to women[3].

1.1.2. Histological Subtypes

The three most common subtypes are[2]:

- Clear Cell RCC, which accounts for approximately 70-75% of all RCC cases and originates from the cells of the proximal tubules of the kidney. The cytoplasm of these cancer cells is clear or pale when viewed under a microscope, due to its high content of lipids and glycogen.
- Papillary RCC makes up about 10-15% of RCC cases and arises from the cells of the distal tubules in the kidney. These tumors form finger-like cell projections on microscopy.
- Chromophobe RCC represents approximately 5% of cases and originates from the intercalated cells of the kidney's collecting ducts. These tumor cells are usually larger and with a distinct halo around the nucleus.

The other histological subtypes account for less than 1% each[2].

The current 2022 World Health Organization (WHO) classification of renal tumors is presented in Table 1.

Table 1. 2022 World Health Organization (WHO) classification of renal tumors

Category	Subtypes
1. Renal Cell tumors	
1.1 Clear cell renal tumors	Clear cell RCC
	Multilocular cystic renal neoplasm of low malignant potential
1.2 Papillary renal tumors	Papillary adenoma
	Papillary RCC
1.3 Oncocytic and chromophobe renal tumors	Oncocytoma of the kidney
	Chromophobe RCC
	Other oncocytic tumors of the kidney
1.4 Collecting duct tumors	Collecting duct carcinoma
1.5 Other renal tumors	Clear cell papillary renal cell tumor
	Mucinous tubular and spindle cell carcinoma
	Tubulocystic RCC
	Acquired cystic disease-associated RCC
	Eosinophilic solid and cystic (ESC) RCC
	RCC NOS (Not Otherwise Specified)
1.6 Molecularly defined renal tumors	TFE3-rearranged RCCs
	TFEB-altered RCC (TFEB-rearranged RCC and TFEB amplified RCC)
	ELOC (formerly TCEB1)-mutated RCC
	Fumarate hydratase-deficient RCC
	Succinate dehydrogenase-deficient RCC
	ALK-rearranged RCCs
	SMARCB1-deficient renal medullary carcinoma
2. Metanephric tumors	
	Metanephric adenoma
	Metanephric adenofibroma
	Metanephric stromal tumor

3. Mixed epithelial and stromal tumor family	
	Mixed epithelial and stromal tumor
	Adult cystic nephroma
4. Renal mesenchymal tumors	
4.1 Adult renal mesenchymal tumors	Classic angiomyolipoma/PEComa of the kidney
	Epithelioid angiomyolipoma/epithelioid PEComa of the kidney
	Renal haemangioblastoma
	Juxtaglomerular cell tumor
	Renomedullary interstitial cell tumor
4.2 Pediatric renal mesenchymal tumors	Ossifying renal tumor of infancy
	Congenial mesoblastic nephroma
	Rhabdoid tumor of kidney
	Clear cell sarcoma of kidney
5. Embryonal neoplasms of the kidney	
	Nephroblastic tumors
	Nephrogenic rests
	Pediatric cystic nephroma
	Cystic partially differentiated nephroblastoma
	Nephroblastoma
6. Miscellaneous tumors	
	Germ cell tumors of the kidney

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1.1.3. Etiology

About 90% of RCC cases are caused by sporadic mutations, while about 10% are hereditary.

Von Hippel-Lindau (VHL) Syndrome is the most common type of hereditary RCC syndrome. It is an autosomal dominant genetic disorder caused by mutations in the VHL gene, located on chromosome 3p25-26[5]. People with VHL syndrome

have an increased risk of developing clear cell RCC, among other tumors in the central nervous system, adrenal glands, and other organs. The VHL gene encodes a protein that helps regulate the stability of hypoxia-inducible factor (HIF), which plays a crucial role in oxygen sensing and cellular responses to low oxygen levels[6]. Mutations in the VHL gene disrupt this regulation, leading to the accumulation of HIF and triggering the development of tumors.

Hereditary Papillary Renal Carcinoma (HPRC) is a rare hereditary form of RCC caused by mutations in the MET proto-oncogene, located on chromosome 7q31.2[5]. Individuals with HPRC have an increased risk of developing papillary type 1 RCC. The MET proto-oncogene encodes a receptor tyrosine kinase involved in cell growth and survival[7].

Birt-Hogg-Dubé (BHD) Syndrome is caused by mutations in the folliculin (FLCN) gene, located on chromosome 17p11.2[5]. BHD patients have an increased risk of developing kidney tumors, mainly chromophobe RCC, as well as lung cysts and skin tumors[8].

Hereditary Leiomyomatosis Renal Cell Carcinoma (HLRCC) is caused by mutations in the fumarate hydratase (FH) gene, located on chromosome 1q42.3-q43[5]. HLRCC patients have an increased risk of developing aggressive forms of type 2 papillary RCC and cutaneous leiomyomas. Mutations in the FH gene lead to a disruption in the tricarboxylic acid cycle, a metabolic pathway that generates energy in cells, leading to the accumulation of fumarate, which triggers tumor development[9].

There are several risk factors for RCC. Some modifiable and fixed risk factors such as obesity, hypertension, smoking, and chronic kidney disease/end-stage kidney disease are well described[2].

1.1.4. Diagnosis

About a third of RCC patients are diagnosed at a metastatic stage and up to 40% of patients treated with curative intent relapse and develop metastasis during follow-up[10].

RCC is usually asymptomatic in its early stages, which can make it difficult to detect. It is estimated to be an incidental diagnosis in more than 60% of cases due to the widespread use of ultrasound and cross-sectional imaging directed at other organ systems[11]. However, as cancer progresses, it can cause a range of symptoms, including hematuria, lumbar or flank pain, fatigue, weight loss, and fever. Only a small percentage of patients with RCC experience the classic triad of symptoms (palpable mass, hematuria, and flank pain)[12]. However, up to 40% of patients with RCC may develop a paraneoplastic syndrome[12]. Anemia is the most common paraneoplastic syndrome, representing 90% of the cases, while weight loss, polycythemia, and hypercalcemia affect 11.8%, 3.6%, and 1.1% of patients with these syndromes, respectively[13].

RCC's diagnosis is based on cross-sectional imaging exams, like contrast-enhanced CT and MRI scans. There is yet no molecular marker that allows the detection or characterization of this type of cancer.

1.1.5. Treatment overview

Treatment for RCC depends on several factors, including tumor stage, nephrometry scores, as well as patient performance status. Surgery is often the primary treatment for RCC and may involve partial or complete removal of the affected kidney.

The preferred surgical approach to localized RCC is a partial nephrectomy (PN), irrespective of the surgical approach. A radical nephrectomy (RN) is still performed in nearly half of the cases and is indicated in locally advanced tumors, tumors with unfavorable locations, and patients with poor performance status.

Most population-based analyses show significantly lower cancer-specific mortality in patients treated with surgery compared to non-surgical management.
Elderly and comorbid patients with incidental small renal masses may have significant competing-cause mortality exceeding RCC-specific mortality. Therefore, in selected patients initial monitoring of small renal masses (active surveillance [AS]), followed, if required, by treatment for progression is appropriate. The

concept of AS differs from the concept of watchful waiting. Watchful waiting is reserved for patients whose comorbidities contra-indicate any subsequent active treatment and who do not require follow-up imaging unless clinically indicated.

For metastatic RCC, treatment is based on targeted immune checkpoint inhibitors (ICI) and/or tyrosine kinase inhibitors (TKI). Several combinations of two ICIs or of an ICI and TKI are approved as the first line in this setting. When the tumor progresses under first-line treatment, patients can be offered subsequent lines of therapy with a multitude of agents and sequencing options. In 2021, there was FDA approval for immunotherapy (with pembrolizumab) in the adjuvant setting for intermediate and high-risk of recurrence RCC patients following radical nephrectomy or following nephrectomy and oligometastasectomy.

1.1.6. Surgical technique

RN was classically performed according to the five principles described by Charles Robson in 1963[14]. These principles were:

1. Early renal pedicle ligation, artery first.
2. Removal of the entire perinephric fat, Gerota's fascia, and overlying peritoneum
3. Adrenalectomy
4. Lymph node dissection
5. *En bloc* resection of the whole specimen, including the kidney, perinephric fat, overlying peritoneum, and lymphatic drainage field.

Robson declares that: "the reason for the first criterion was obvious in that these tumors are notorious for spread via the bloodstream and therefore, the renal pedicle should be occluded before any extensive manipulation of the tumor occurs."[14]

The last four principles have been rebutted and have been abandoned.

Routine adrenalectomy is not indicated if preoperative imaging and intraoperative findings show no tumor invasion. Lymphadenectomy has consistently failed to provide a survival benefit in the literature and is reserved for staging purposes in

patients with enlarged lymph nodes. With the development of PN and its robust evidence base of similar oncological outcomes as RN, the second and fifth principles have also fallen.

However, the first principle of early ligation of the renal pedicle is still standing since no research has yet provided evidence.

A few publications have described a technique for early dissection and ligation of the renal pedicle[15–17]. Porpiglia et al. have described the technique of direct dissection of the renal artery in transperitoneal laparoscopic RN, concluding that there was no difference in perioperative and survival outcomes in a follow-up period ranging from one to 46 months[15]. However, to our knowledge, there are no publications in the literature that explore the concept of a no-touch RN for the surgical approach of KC.

In most cases, minimally invasive procedures such as laparoscopy or robotic-assisted laparoscopy are offered when available. Robot-assisted laparoscopic and conventional laparoscopic PN or RN are associated with shorter lengths of hospital stay, lower blood loss, and less postoperative pain compared to open surgery[18]. A systematic review comparing robot-assisted laparoscopic vs. conventional laparoscopic RN, revealed no substantial differences in functional or oncological outcomes[19].

1.1.7. Prognosis

Considerable progress has been made in the treatment of patients with renal cell carcinoma, with innovative surgical and systemic strategies revolutionizing the management of this disease. However, the early diagnosis of RCC remains a challenge and the lack of diagnostic tools to assess progression and metastasis significantly influences the survival of RCC patients.

RCC prognosis varies depending on several factors, which can be classified as anatomical, histological, clinical, and molecular. Anatomical prognostic factors are included in the TNM classification system: tumor size, venous invasion or extension, collecting system invasion, perinephric and sinus fat invasion, adrenal

invasion, and lymph node or distant metastasis[20]. Histological factors comprise tumor grade, RCC histological subtype, lymphovascular invasion, tumor necrosis, and collecting system invasion[21] [180,181]. The WHO/ISUP tumor grade is considered one of the most important prognostic factors[22]. Clinical prognostic factors include performance status, cachexia, anemia, platelet counts, neutrophil counts, lymphocyte counts, C-reactive protein (CRP), albumin, and neutrophil-to-lymphocyte ratio (NLR)[23–29]. Cancer-specific survival figures according to stage are presented in Table 2.

Table 2. Cancer-specific survival according to stage

Grade	HR (95% CI)
T1N0M0	Reference
T2N0M0	2.71 (2.17–3.39)
T3N0M0	5.20 (4.36–6.21)
T4N0M0	16.88 (12.40–22.98)
N+M0	16.33 (12.89–20.73)
M+	33.23 (28.18–39.18)

1.2. RCC BIOMARKER RESEARCH

An unmet need persists for a clinically validated and useful RCC biomarker capable of facilitating early diagnosis, detecting micro metastases, enhancing prognostication, aiding in treatment selection, and monitoring, and detecting disease relapse.

Current RCC biomarker research is focusing on liquid biopsies. The rationale behind liquid biopsies is to obtain biological material originating from the tumor with a simple blood sample. The purpose is to access phenotypic and genetic data of the tumor, without the invasion of a tumor or metastasis biopsy.

This can not only allow non-invasive early cancer diagnosis, but also sequential sampling during disease management, enabling better treatment decisions, monitoring of treatment response, and providing a more accurate prognosis.

Liquid biopsy can retrieve different types of circulating biomarkers, such as circulating tumor DNA (ctDNA), micro RNA (miRNA), and CTCs[30–33].

1.3. RESEARCH ON CTCs

One of the most promising developments in translational cancer medicine has been the discovery and study of CTCs as a minimally invasive multifunctional biomarker. The first observation of CTCs in the peripheral blood of a patient with metastatic cancer from an unknown primary site was made by Thomas Ashworth in 1869[34]. Technological advances have been improving our ability to isolate as well as harness more knowledge of these quite rare cells, which have led to an improved understanding of basic cancer biology.

CTCs constitute an exceedingly small fraction of cells relative to a background of 1 million white blood cells (WBCs) and 1 billion red blood cells (RBCs) per milliliter of peripheral blood[35]. CTCs are released from tumors and enter the bloodstream early before metastasis occurs. CTCs and CTC clusters are seen as the intermediate stages of metastasis[36]. They enter the circulation by either passive cellular shedding from the primary tumor site or through a dynamic process that comprises stromal invasion and subsequent intravasation into the bloodstream[37,38]. Within the circulation, most CTCs will not be able to form distant metastasis. CTCs must survive shear stress, anoikis, and evade the host immune system to extravasate at a distant site[37]. Only a minute fraction of CTCs can survive, and only 0.01% are considered to be of high metastatic potential[39]. Once at their new location, CTCs must adapt to the local microenvironment, where they can lay dormant in a quiescent state or undergo proliferation to develop into metastatic foci[40]. CTCs can also originate from metastatic sites, rather than just primary tumors[40].

CTCs' clinical potential has been explored in several cancer types[41]. CTC counts have proven to be an independent prognostic marker for metastatic breast cancer and a surrogate marker of recurrence and disease progression[42,43]. Within the field of urologic oncology, CTCs have been explored as biomarkers of prostate, bladder, and kidney cancer[40]. In metastatic castration-resistant prostate cancer and metastatic colorectal cancer, an association has also been shown between CTC counts and overall survival (OS), cancer-specific survival (CSS), and progression-free survival (PFS)[44,45].

1.4. CTC RESEARCH IN RCC

The heterogeneous, aggressive natural history and propensity for recurrence of kidney cancer instigate the need for improved individualization of care using circulating tumor cells.

Successful management of RCC patients, particularly those with recurrent and metastatic RCC (mRCC), largely relies on early detection of disease progression. This detection is currently limited to identifying overt enlargement of lymph nodes and metastatic nodules. The threshold for identifying a renal mass or lymph node metastasis is approximately 1 cm, which implies that we can only detect the disease when more than 100,000,000 cancer cells have developed[46]. CTCs have the potential to detect the disease in an earlier stage, while it is still in a subclinical phase.

CTC studies in RCC have been very limited when compared to a large number of studies in colon, breast, and prostate cancer. This is due mainly to the fact that there are no appropriate surface markers that can be used to capture RCC CTCs.

The initial work on CTC detection in RCC focused on indirect RT-PCR-based detection approaches. In studies published in the 1990s, Pontes et al.[47] and Hioki and Sugimura[48] used RT-PCR to detect circulating tumor DNA in patients with RCC. In another study, Ashida et al. identified mutations in the von Hippel-Lindau gene in circulating DNA using RT-PCR[49]. McKiernan et al. used the same method

to detect carbonic anhydrase IX (CA9) expression in peripheral blood, a gene that is ubiquitously expressed by the clear cell subtype of RCC (ccRCC)[50]. They tested 36 patients with ccRCC (27 with localized RCC and nine with metastatic disease) and found a detectable signal in 50% (18) of them. The assay had high specificity, with signal detection in zero of five patients with a benign renal tumor and in only one of 55 (1.8%) healthy donor controls. These studies were all limited by the low detection rate of RCC cell mRNA in peripheral blood.

Direct CTC detection and isolation only came later, with the appearance of cellular-based assays like immunomagnetic detection methods[51]. In small studies, CTCs were detected in approximately 30% to 92% of patients[52]. In the largest study of CTCs in RCC, Bluemke et al. used the AutoMACS immunomagnetic cell separation system and immunocytochemical staining of cytokeratin 8/18 to detect CTCs in 52,6% of patients (81 of 154)[53]. CTC detection at initial diagnosis was an independent prognostic factor that correlated with overall survival (RR 2.3; $p = 0.048$), lymph node invasion ($p < 0.001$), and distant metastasis ($p = 0.014$)[53].

A semi-automated immunomagnetic platform called CellSearch® (Veridex, LLC, Raritan, NJ, USA), which uses epithelial cell adhesion molecule (EpCAM) as an epithelial cell marker, was the first FDA-approved platform for CTC analysis in 200454. In the initial validation report of the CellSearch® system, blood from 11 mRCC patients was tested, with a mean of only one CTC detected and only 25% of patients having ≥2 CTCs54. This was the first time CTC counts were studied, as previous studies had reported only detection rates. Likewise, Gradilone et al. reported detecting ≥1 CTC with the CellSearch® test in only 16% of patients with metastatic RCC55. This low frequency of CTCs detected in studies using the CellSearch® is likely explained by the observation that only 20–40% of RCC express EpCAM and thus it is not an ideal capture antigen for the detection of CTCs in RCC patients56–58.

However, only 18.6% of RCCs express EpCAM which has led researchers to try alternative approaches for CTC detection[59]. The lack of EpCAM expression in RCC has led researchers to try alternative approaches for CTC detection. Liu et al. used a microfluidic device (NanoVelcro® chip) composed of a chaotic mixer and a

nanowire substrate coated with antibodies, in this case, for carbonic anhydrase 9 (CA9) and CD147[59]. CA9 is a tumor hypoxia cell membrane marker expressed in many types of human cancer and overtly expressed in RCC[60]. CD147, a highly-glycosylated member of the immunoglobulin superfamily expressed on the surface of many malignant tumors, is also a cell membrane tumor marker implicated in the metastatic process by enhancing epithelial-to-mesenchymal transition (EMT) cell invasion through activation of the MAPK/ERK pathway[61]. Before implementing this positive-selection method, the investigators validated the expression of these two markers in ccRCC tissue specimens, demonstrating that, when CA9 and CD147 are used in combination, 97.1% of tumors were positive for these markers, in sharp contrast to their observation that only 18.6% of tumors expressed EpCAM[59]. Using these markers for positive selection, instead of EpCAM, they could detect CTCs in 94.7% (72 of 76) of patients with ccRCC, with a mean of 20 cells isolated, 7.5 times more than in normal controls (patients with benign renal tumors). No cells were detected in 13 of 15 (86.7%) healthy donors. A correlation of CTC counts with staging was found, with 8.76 ± 4.11 CTCs for stage I RCC, 9.32 ± 3.64 for stage 2, 18.56 ± 5.96 for stage 3, and 20.42 ± 5.97 for stage 4. This represented a 2.2-fold increase in CTC counts between late-stage (stages 3 and 4) and early-stage (stages 1 and 2) disease ($p<0.001$). In the same study, there was a positive correlation between vimentin-positive mesenchymal CTC counts and stage ($p < 0.001$). This 2016 paper was the first to show satisfactory detection rates and to accurately measure CTC counts.

Other studies found a correlation between CTC detection and survival outcomes. A prospective study showed that CTC-positive metastatic RCC patients had a significantly lower progression-free survival (PFS) rate (hazard ratio [HR] 4.17, 95% confidence interval 1.41-11.99, p=0.01) but no difference in overall survival62. Additionally, CTC presence predicted a poorer response to tyrosine kinase inhibitors (TKI) (87.5% vs. 37.1%, p=0.01). In a large prospective study with 195 patients, the ones with ≥3 CTCs had shorter OS compared to the ones with <3 CTCs (13.8 vs. 52.8 months, HR 1.99, p=0.003)63.

1.5. CTC DETECTION TECHNIQUES

Many platforms for CTC enrichment, detection, and characterization have been investigated over recent years, reflecting the multitude of potential research and clinical applications for a system able to overcome current limitations[64,65]. CTC detection usually encompasses three steps: CTC enrichment, CTC isolation and detection, and CTC characterization, for instance through FISH or gene expression profiling[66]. There is currently no standardized detection method for CTCs.

A proportion of these cells is lost during processing, hindering their detection and making the process utterly dependent on the sensitivity and specificity of the technology used[67]. CTC detection rates (DR) have been relatively poor and there is an ongoing search for a method that is both sensitive, specific, reproducible, and inexpensive.

A study comparing different isolation techniques for RCC CTC detection has concluded that the most efficient is the cell-size-based approach[68].

The great heterogeneity of techniques and results calls for a systematic review of the literature to structure the findings so far and to guide the research project.

1.6. CTC EMT PROCESS

The EMT process is a normal cell-biological program that occurs in various tissue types and circumstances[69]. It converts epithelial cells along the epithelial-mesenchymal axis towards a more mesenchymal phenotype. These cells develop intermediate phenotypic states along the referred axis. With sufficient drive, a fully epithelial cell can be converted to a fully mesenchymal cell[70]. Epithelial cells are characterized by cell-to-cell junctions and apical-basal polarity, whereas mesenchymal cells exhibit no polarity, a roundish or spindle-like morphology, higher motility, and invasiveness potential[70,71]. Cells undergoing EMT express mesenchymal markers, such as vimentin, N-cadherin, O-cadherin,

fibronectin, and twist[72,73]. They also lose EpCAM, CK, E-cadherin, and other epithelial markers[69].

The EMT program has a crucial role in various processes, including embryogenesis, wound healing, fibrosis, and cancer progression[70]. In the process of wound healing, epithelial cells lying on the wound edges undergo a partial EMT, gaining the necessary motility to coordinately bridge the edges[74]. After wound closure, these cells then revert to their epithelial phenotype through an inverse process of mesenchymal-to-epithelial (MET) changes[74].

The role of EMT in tumor progression has been revealed in numerous studies in a multitude of cancers of epithelial origin[69]. EMT enables local invasion of cancer cells in the primary tumor, their intravasation into blood vessels, extravasation into distant sites, and survival in the new cell environment to form micrometastasis[75]. Research in mouse and patient-derived xenograft models has shown that mesenchymal cells colonizing distant sites need to undergo MET in order to actually form metastases[76]. Evidence also indicates that EMT confers multidrug resistance to cancer cells[77].

Data has shown that a big proportion of RCC cells undergo early EMT, developing resistance to anoikis and other apoptotic signals[78,79]. As previously stated, this makes CTC detection in RCC patients more challenging than in other carcinomas.

1.7. CTC RELEASE IN RCC SURGERY

CTCs have been even less studied in the RCC surgical context. Some exploratory studies have been done on perioperative CTCs in the RCC surgical context.

Ashida et al., in 2000, found that cancer cells were released into the circulation during and after surgery[49]. They inferred the CTC release through nested RT-PCR detection of mutations of the VHL gene, before and after radical nephrectomy both in peripheral and renal venous blood samples. CTCs were identified in 2 of 17 (11.2%) patients before surgery, and 6 of 16 (37.5%) patients within 24 h after

surgery. The proportion of patients with detectable CTCs decreased to 3 of 16 (18.8%) on postoperative day 7 and 2 of 11 (18.2%) patients on postoperative day 303. 75% (15 of 20) of patients had CTCs detected in renal vein blood samples in 15 of 20 (75%) patients. Furthermore, 7 of 9 (77.8%) patients presented the mutation in the renal vein samples. These results showed that intraoperative tumor manipulation enhanced RCC cell dissemination.

In 2006, Ohlmann et al., found that the detection rate of CTCs (using RT-PCR for CA9) increased from 46% (11/24) before surgery to 58% (14/24) after surgery[80].

Bluemke et al., in 2009, also found an increase from 37.1% (52/140) to 45.4% (35/77) between pre-operative (at diagnosis) and 5 to 7 days postoperative peripheral blood samples, respectively[53]. They found that this increase resulted in a rise from 2.7 (p=0.049) to 4.3 (p=0.036) in the relative risk of death, compared to patients without CTCs. Overall, the presence of CTCs represented a 2.3 (p=0.048) RR of death. The median follow-up time was 15 months (1-56 months).

In 2013, El-Heliebi et al. published a paper that also showed an increase in the detection rate of CTCs after surgery, identified by cytomorphological criteria and defined as cellular aggregates of non-hematological cells (CNHC)[81]. Comparing the days before and after surgery, detection rates for CNHC-MF increased from 26% to 40% (p = 0.040), for CNHC-UMF from 34% to 43% (p = 0.045), and for CNHC-BF from 21% to 43% (p = 0.004).

Two more recent studies, using more sensitive CTC capture techniques, not only reported a 100% detection rate before and after surgery in localized RCC patients but also provided CTC counts[82,83]. In a retrospective study on 69 patients who underwent RCC surgery, Wang et al. showed that metastatic patients showed a progressive increase in CTC numbers, from 7.71 ± 3.82 at baseline to 11.65 ± 1.99 at 6 months ($p<0.05$) and 16.60 ± 8.25 at 12 months ($p<0.05$)[82]. In contrast, non-metastatic patients showed a decrease in CTC numbers at 12 months, from 8.43 ± 5.15 at baseline and 8.93 ± 4.74 at 6 months to 6.82 ± 2.68 at 12 months ($p<0.05$)[82]. A prospective cohort study observed a higher immediate postoperative CTC number after open radical nephrectomy (ORN), with an increase from 7.7 ±

6.8 to 22.5 ± 26.3 ($p<0.05$), but not after laparoscopic radical nephrectomy (LRN), open PN (OPN), or laparoscopic PN (LPN)[83]. They also found that tumor diameter significantly correlated with postoperative CTC counts ($r = 0.30$, $p = 0.01$), but not with preoperative CTC counts, and TNM staging did not impact CTC release during surgery[83].

1.8. THE CONCEPT OF NO-TOUCH ONCOLOGIC SURGERY

CTC release during surgical manipulation may be an important marker of surgical quality and may impact survival.

The concept of no-touch tumor resection was introduced in colorectal cancer surgery in 1977[84].

The no-touch colon resection technique proved to reduce CTC release (inferred from RT-PCR targeting specific tumor mutations) from 73% to 14%, compared to a conventional technique ($p=0.05$)[85]. No outcome data were analyzed. However, two RCTs have failed to demonstrate a significant difference in the outcomes of a no-touch surgical technique in tumor resection in colon cancer[86,87].

In a pilot comparative study in pancreatic cancer surgery, the recurrence rate was 90% vs. 38% ($p=0.043$), and the mean survival time was 21.2±5.8 vs. 41.5±5.6 months ($p=0.018$) for the conventional and no-touch groups, respectively[88].

In a study on cervical cancer, a no-touch laparoscopic radical hysterectomy showed better 5-year DFS (adjusted hazard ratio [HR], 0.202; 95% confidence interval [CI], 0.069-0.594; $p=0.004$) and 5-year OS (adjusted HR, 0.163; 95% CI, 0.035-0.748; $p=0.020$) compared with a conventional technique[89].

In a prospective lung cancer trial, CTC detection after surgery was significantly lower in a no-touch pulmonary wedge resection compared to a conventional approach (12.5 vs. 85.7%, $p=0.02$, respectively)[90].

As mentioned before, four of the five Robson principles of RN have been rebutted and abandoned, leaving a last one standing, which is the early ligation of the renal pedicle. This is the basis for the no-touch concept in RN. To our knowledge, there are no publications addressing the no-touch technique for RN.

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2. OBJECTIVES

The present doctoral thesis aims to deepen the knowledge and role of CTCs in RCC patients. The work is the result of a long research process, which involved the review of existing literature, the collection, and analysis of empirical data, as well as critical reflection on the results obtained.

In this work, we aimed:

1. To perform a systematic review of the literature on the methods used in CTC enrichment and detection in RCC patients, their detection rates and CTC counts and characterization, as well as CTCs' role as biomarkers of diagnosis, staging, prognosis, and treatment response.

The great heterogeneity of techniques and results called for a systematic review of the literature to structure previous findings and guide the subsequent steps of this research project.

2. To test and validate the RUBYchip™ microfluidic size-based device for CTC isolation and analysis in RCC patients.

To accomplish this goal, we devised an in vitro validation protocol with three different RCC cell lines, with blood processing and staining optimizations, as well as spiking experiments. For clinical validation, samples from 18 patients with different disease stages were analyzed.

3. To determine if surgical manipulation of the kidney during RN increases CTC release and if CTC release can be reduced during RN by using a no-touch technique, with an early renal pedicle ligation, compared to a conventional technique.

To answer these questions, we devised a prospective randomized controlled trial comparing the two surgical techniques. Secondary outcomes were to analyze if this reduction influences survival outcomes, to describe the CTC profile in RCC patients, and to correlate CTC counts with clinicopathological and imagiological parameters.

3. RESEARCH ARTICLE: Circulating tumor cell detection methods in renal cell carcinoma: A systematic review

3. TITLE: Circulating tumor cell detection methods in renal cell carcinoma: A systematic review

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3.1. ABSTRACT

Circulating tumor cells (CTCs) have a potential role as the missing renal cell carcinoma (RCC) biomarker. However, available evidence is limited, and detection methods lack standardization, hindering clinical use. We performed a systematic review on CTC enrichment and detection methods and its role as a biomarker in RCC. Full-text screening identified 54 studies. Reviewed studies showed wide heterogeneity, low evidence level and high risk of bias. Various CTC detection platforms and molecular markers have been used, but none has proven to be superior. CTC detection and CTC count seem to correlate with staging and survival outcomes, although evidence is inconsistent. CTC research is still in an exploratory phase, particularly in RCC. Further studies are still necessary to achieve a standardization of techniques, molecular markers, CTC definitions, and terminology. This is essential to ascertain the role of CTCs as a biomarker and guide future liquid biopsy research in RCC.

KEYWORDS:
Biomarker; Circulating tumor cells; Liquid biopsy; Metastases; Renal cell carcinoma

3.2. INTRODUCTION

The presence of metastases is the most important prognostic factor in cancer patients.[1,2] One third of patients with renal cell carcinoma (RCC) present with regional or metastatic disease and up to 40% of those treated for localized disease ultimately develop metastases or, less frequently, local recurrence.[3–5] Unfortunately, RCC is still diagnosed based on the identification of bulky tumor masses on cross-sectional imaging. There is currently no biomarker capable of detecting metastases or even localized disease at early disease stages, before gross masses develop.[6] To improve RCC patients' survival, it is paramount to identify a biomarker able to accurately diagnose both early localized and micrometastatic disease.

Liquid biopsies, namely circulating tumor cells (CTCs), are an emerging non-invasive tool for diagnosing and monitoring disease status in several urogenital cancers.[7] In 1869, Ashworth was the first to report the presence of cells similar to the primary tumor in *post-mortem* blood samples.[8] CTCs are tumor cells which have migrated from a primary tumor or metastatic site through passive shedding or by the dynamic process of stromal invasion and resultant intravasation into the bloodstream.[9,10] Only about 0.01% of CTCs are thought to form metastases at remote sites.[11]

The potential clinical value of CTCs has been explored in several tumor types. It has been shown that CTC count (CTCn) is an independent prognostic marker for metastatic breast cancer and a predictor of recurrence and disease progression. [12,13] In metastatic castration-resistant prostate cancer and metastatic colorectal cancer, an association has also been shown between CTCn and overall survival (OS), cancer-specific survival (CSS), and progression-free survival (PFS).[14,15]

CTC analysis typically includes three steps: 1) blood sample preparation and CTC enrichment; 2) CTC isolation and detection; and 3) further CTC characterization, for instance through gene expression profiling.[16]

There is currently no standard detection method for CTCs. These are extremely rare compared to whole blood cells, with an estimated CTCn of one CTC per billion normal blood cells in metastatic cancer patients.[17] Additionally, a proportion of these cells is lost during sample processing, making their detection difficult and utterly reliant on the sensitivity and specificity of the methods used. Furthermore, the lack of specific RCC molecular markers has hindered CTC research in this tumor compared to other epithelial tumors. Previously reported CTC detection rate (DR) is relatively poor and there is an ongoing search for a method that is highly sensitive and specific, while at the same time reproducible, inexpensive, and simple to use.

Cellsearch® is the only FDA-approved CTC detection platform. However, it is deemed inappropriate for RCC due to the lack of epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) expression in many CTCs that have undergone epithelial-to-mesenchymal transition (EMT).[18] Data suggests that many RCC cells undergo early EMT, with the development of resistance to anoikis and other apoptotic signals.[16,19] This feature is illustrated by a gain in the expression of mesenchymal markers, such as vimentin.[20]

The great heterogeneity of techniques and results calls for a systematic review of the literature to structure current findings and possibly guide future research to improve knowledge of CTCs in RCC and their clinical applicability. Herein are described CTC enrichment and detection methods used so far, their DR and CTCn, and available evidence on their value in diagnosis, staging, prognosis, and treatment response.

3.3. EVIDENCE ACQUISITION

This systematic review of published literature was performed in accordance with the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) guidelines for systematic reviews.[21] Methods and inclusion criteria were stated in advance and documented in a protocol registered in the

International Prospective Register of Systematic Reviews (PROSPERO) International Registry — CRD42017074226.

Literature search was conducted on Embase®, Web of Science®, Ovid MEDLINE®, Ovid MEDLINE® Daily and Ovid MEDLINE® Epub Ahead of Print, and In-Process & Other Non-Indexed Citations, from inception until April 1, 2020. The complete search string is attached as supplementary material.

Literature search aimed to include published peer-reviewed articles, conference proceedings, and other gray literature. Inclusion criteria comprised studies describing CTC enrichment and detection methods from peripheral or central blood samples of RCC patients of any histology and staging. Publications in human adult patients (>18 years) with a sample size larger than one patient were considered. Editorial comments, letters to the editor, and review articles were excluded.

Title and abstract screening followed by full-text screening were independently conducted by three authors. Duplicates were excluded. Disagreement between authors was resolved by a fourth author. Data extraction was performed by four authors. The following data items were retrieved: study reference, study design, sample size, participant characteristics, enrichment techniques, and detection methods. Data on CTC performance as a diagnostic (CTC DR and CTCn), prognostic (OS, CSS, and PFS), and treatment response biomarker (post-op CTC DR and CTCn) according to the BEST (Biomarkers, EndpointS, and other Tools) approach was also collected.[22]

Due to the exploratory nature and heterogeneity of selected studies, two different critical appraisal tools to assess risk of bias were applied according to study design. For observational descriptive studies, the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Case Series was used.[23] For observational analytical (cohort and case-control) studies, the Newcastle-Ottawa Quality Assessment Scale was applied.[24] (Appendix — tables 4.1 and 4.2)

Given the great heterogeneity in detection methods, DRs, populations, and outcomes between studies, a quantitative pooling of study results was not appropriate, and hence a qualitative analysis was performed.

3.4. EVIDENCE SYNTHESIS

The literature search identified 2529 studies, 54 of which were included after screening and eligibility assessment (Figure 1).

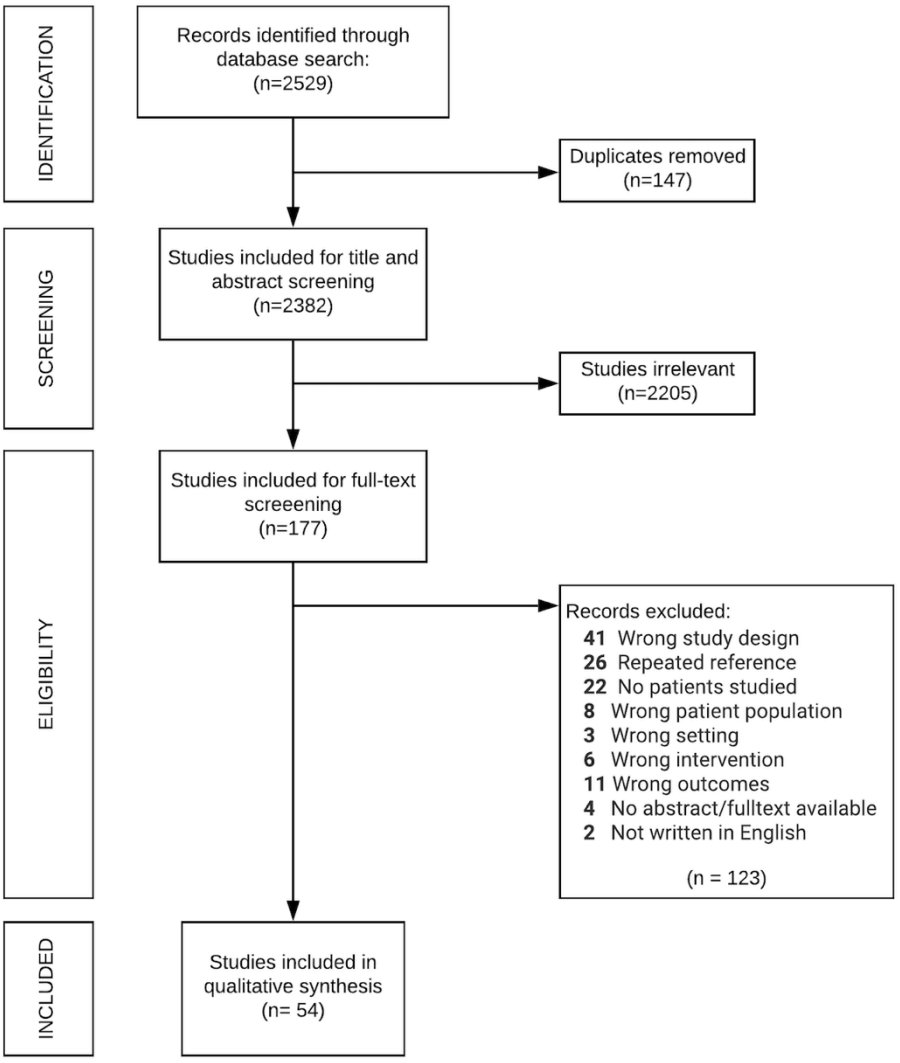

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flow diagram

Retrieved evidence was organized into three topics: CTC enrichment techniques, CTC detection methods, and CTC performance as a diagnostic, prognostic, and treatment response biomarker (Figure 2).

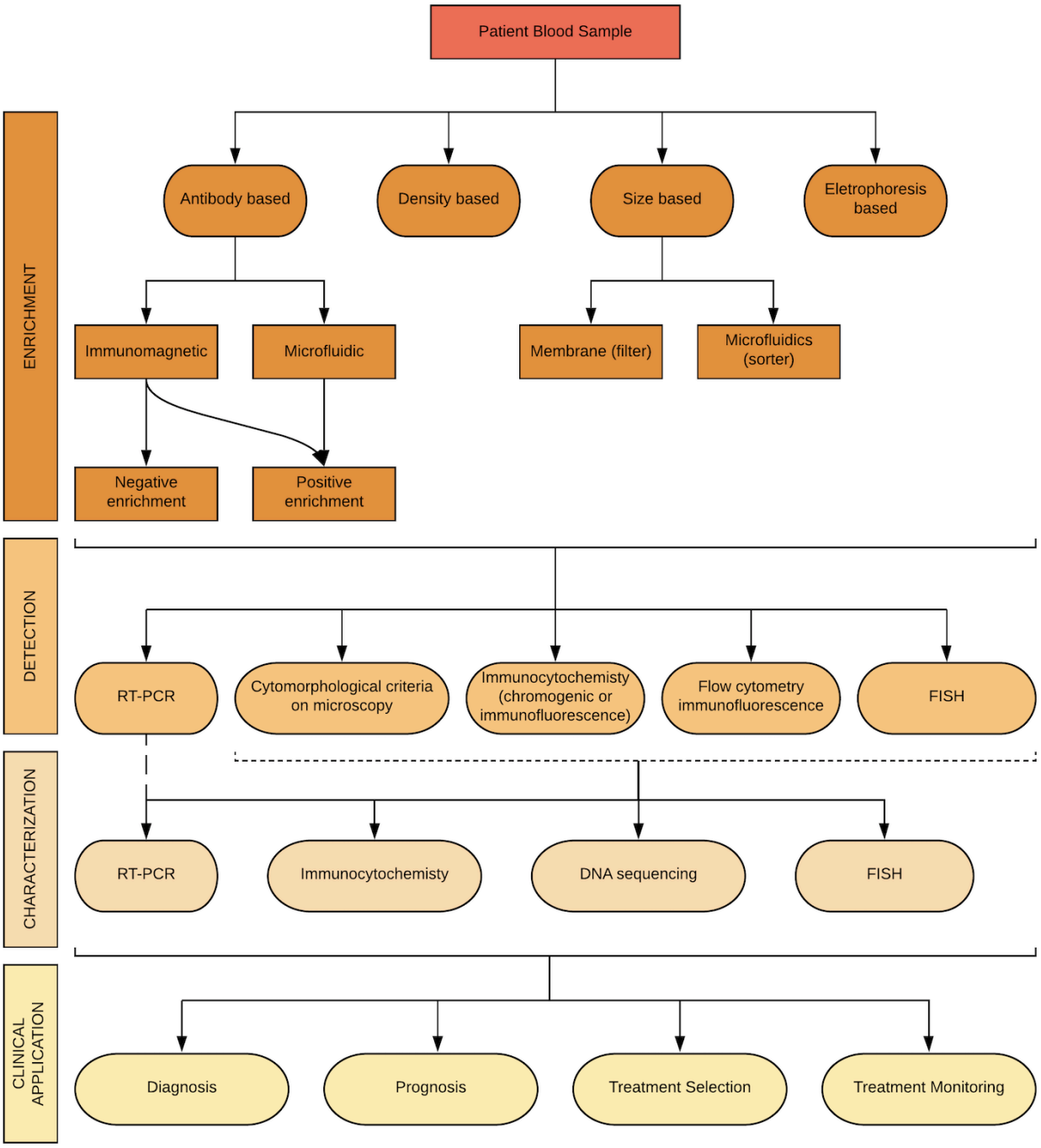

Figure 2. CTCs enrichment, detection, and characterization methods flow chart and their potential role as a biomarker

3.4.1. CTC enrichment methods

Enrichment refers to the process of concentrating these very rare cells in a sample for subsequent detection and characterization. This can be done in two ways: by positive enrichment, in which CTCs are isolated or selected from the sample, or by negative enrichment, in which the sample is depleted of blood cells.

A multitude of enrichment methods has been used to date, which can be organized in four different categories: antibody- (27 studies), density- (14 studies), size- (11 studies), and electrophoresis- (1 study) based methods (Table 1 and Figure 2).

3.4.1.1. Antibody-based methods

Antibody-based enrichment methods usually rely on immunomagnetic selection. For negative enrichment, CD45 is the marker typically applied for leukocyte depletion. A commonly used technique is Magnetic Cell Separation System (MACS), which couples ferromagnetic nanoparticles (magnetic beads) to CD45 antibodies.[11,25–28]

The most commonly used markers for positive enrichment are EpCAM and CKs due to the epithelial origin of RCC.[29] CellSearch® uses ferrofluid nanoparticles coated with anti-EpCAM antibodies to retain CTCs passing through a magnetic field.[30–38]

One author used a microfluidic antibody-based device consisting of chips with geometrically arranged microspots that promote cell-antibody binding. This device showed higher CTC capture efficiency in RCC patients with a modified NanoVelcro platform using CA9 and CD147-capture antibodies.[18]

3.4.1.2. Density-based methods

This technique is based on the fact that tumor cells and leukocytes have different densities, enabling their separation by centrifugation. This method was mostly used in initial studies and enables a marker-independent enrichment, yet leading to tumor cell loss and false negatives.[6,39–51]

3.4.1.3. Size-based methods

This label-free cell selection method is based on the bigger size and less deformability of CTCs compared to blood cells. It has the advantage of minimizing cell loss. It also allows the sorting of viable cells for further downstream analysis. The main limitations are the risk of filter/channel clogging due to cell agglutination and losing CTCs smaller than pore sizes. Size-based enrichment can be accomplished through membrane-based [10,38,52–57] or microfluidic devices [58–60], most commonly calibrated to capture cells bigger than 8 µm.

3.4.1.4. Electrophoresis-based methods

Only one article reported the use of an electrophoresis-based microfluidic positive enrichment method, the ApoStream® Rare Cell Enrichment Platform. It relies on the fact that CTCs have specific polarization charges due to different diameters, membrane area, density, conductivity, and volume. Hence, CTCs are attracted to the bottom of the device in an electrical field, while the sample flows.[61]

Table 1. Studies assessing CTCs in RCC patients - CTC Detection Rates and Counts

Reference (author/year)	Enrichment / Isolation Method	Detection Method	Molecular Markers	Patients (n)	Staging (% M1)	CTC DR (%)	CTCn (median, [range], units)
Glaves 1988	Density based - DGC	Immunocytochemical (IF)	CK	10	30	80.0	160.5 [0-7309]/mL*
Hioki 1999	Density based - DGC	RT-PCR	CK19	19	21	47	-
Uemura 1999	Density based - DGC	RT-PCR	CA9	42	NA	76.2	-
McKiernan 1999	Density based - DGC	RT-PCR	CA9	42	21.4	49.0	-
Ashida 2000	Density based - DGC	RT-PCR	VHL	20	15	75.0	-
de la Taille 2000	Density based - DGC	RT-PCR	CA9, PSMA	59	4.0	18.6	-
Bilkenroth 2001	Antibody based - IM(-)	Immunocytochemical (CG)	CK	59	13.6	32.2	8.0 [1-38]/8mL
Meye 2002	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK	24	16.7	41.7	5.0 [1-13]/16mL*
Fehm 2002	Antibody based - IM(-)	Immunocytochemical (IF)	CK	4	0	100.0	-
Uemura 2003	Density based - DGC	RT-PCR	CA9	38	31.6	18.5	-
Shimazui 2003	Density based - DGC	RT-PCR	Cadherin 6	87	13.8	OA 52.9 M0 45.0 M1 70.4	-
Shimazui 2004	Density based - DGC	RT-PCR	Cadherin 6	66	34.8	OA 47.0 M0 34.9 M1 69.9	-
Allard 2004	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	11	100	-	1±1/7.5mL
Blumke 2005	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK or CK8/18	214	NA	37.4	0.56 [0.06-4.75]/mL
Li 2005	Density based - DGC	RT-PCR	Cadherin 6	46	23.9	45.7	-
Ohlman 2006	Not done	RT-PCR	CA9	24	0	45.8	-
Lomo 2006†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	10	NA	-	2.6(±1.4) [1-5]/7.5mL

Gilbert 2006	Density based - DGC	RT-PCR	CA9	36	0	33.3	-
Bluemke 2009	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK or CK8/18	154	NA	37.1	6.0 [1-51]/16mL
Seideman 2009†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	12	NA	92.0	-
Gutschi 2010†	Size based - MB	Immunocytochemical (CG)	-	19	21	63.2	-
Gradilone 2011	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19 CD44	25	100	16.0	1.0 [1-4]/2mL
Iacovelli 2011†	Antibody based - IM	RT-PCR	CK8, Vimentin	18	100	50.0	-
Rossi 2012	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	53	13.2	58.5	2.0 [1-141]/7.5mL
Williams 2012†	Size based - MB	-	-	7	NA	100.0	[1-128]
Mittal 2012†	Size based - MB	Immunocytochemical (IF)	CK	26	100	50.0	[0-58]/mL
Ramirez 2012†	Density based - DGC	Immunocytochemical (IF)	EpCAM, CA9, CD10, EGFR	4	100	75.0	[1-6]/3mL
El-Heliebi 2013	Size based - MB	Cytomorphological analysis Immunocytochemical (CG)	CA9	30	3.3	29 CA9 16.2	3.0 [1-62]/8mL
Yip 2014†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	55	100.0	16.1	-
Mittal 2014†	Size based - MB	Immunocytochemical (IF)	CK	17	100	53.0	OA 1/mL
Theil 2014†	Antibody based - IM(+)	Immunocytochemical (CG)	EpCAM, CK, MUC1	20	NA	91.6	M0 5.3 [0-11]/7.5mL M1 9.6 [2-44]/7.5mL EpCAM+ 6.7 [2-15]/7.5mL EpCAM+/MUC1+ 5.1[1-8]/7.5mL
Gorin 2015†	Electrophoresis based - Microfluidic	FISH	VHL gene	30	100	26.7	-
Novikova 2015†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	6	100	67.0	[2-5]/7.5mL
Amato 2015†	Density based - DGC	Cytomorphological analysis Immunocytochemical (IF)	CK	4	100	100.0	1.6/mL 9.1/mL

Ionescu-Zanetti 2015†	Antibody based - IM(+)	Flow cytometry IF	EpCAM	6	NA	100.0	248 [14-731]/15mL*
Tseng 2015†	Antibody based - IM(+)	Flow cytometry IF	EpCAM	8	100	100.0	260 [65-1413]/15mL*
Liu S. 2016	Antibody based - IM(+)	Immunocytochemical (IF) Cytomorphological analysis	CK, CA9, CD147, vimentin	76	15.8	94.7	12.8 (±6.9)/2mL
Nel 2016	Antibody based - IM(-)	Immunocytochemical (IF)	CK,n-cadherin, CD133	14	100.0	64.3	7.2/1000 PBMNC
Zhang 2016	Antibody based - IIM(+)	Immunocytochemical (IF)	c-MET, CK	10	100	10.0	0 [0-3]/7.5mL
Desotelle 2016†	Not reported	Flow cytometry IF	EpCAM, CA9, CA12, PAX8, CK	20	NA	-	CK+/CA9+ 24.5 [0-5410]/7.5mL CK+/EpCAM+ 5.5 [1-231]/7.5mL
Liu M. 2017	Density based - DGC	Cytomorphological analysis Acridine orange fluorescence	-	139	19.4	OA 13.67 M0 8.9 M1 33.3	-
Arafat 2017†	Antibody based - IM(+)	Immunocytochemical (IF)	CA9,CA12, PAX8	27	100	96.2	-
Basso 2017†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	195	100	46.6	2 [1-263]/7.5mL
Broncy 2018	Size based - MB	Cytomorphological analysis DNA amplification nested PCR	-	30	6.7	96.7	-
Xing 2018	Antibody based - IM(+)	Immunocytochemical (IF)	CK	7	42.9	57.1	1.29 [0-3]/mL*
Bai 2018	Antibody based - IM(+) Size based - MB	Immunocytochemical (IF) Cytomorphological analysis	CK8, CK18, CK19 -	36	27.8	36.1	-
Kang 2018	Size based - Microfluidics	Immunocytochemical (IF) Cytomorphological analysis	EpCAM, CK, CD10	48	33.3	27.1	1 [0-5]/mL *
Ye 2018	Antibody based - IM(-)	FISH Immunocytochemical (IF)	CEP8, CK18, EpCAM	74	NA	91.9	8 [1-52]/7.5mL
Wang 2019	Size based - MB	RNA FISH	EpCAM, CK8, CK18, CK19, Vimentin, Twist, Beclin-1	69	0	100.0	Epithelial 8.21(±4.77)/7.5mL Mesenchymal 2.30(±1.41)/7.5mL Mixed 5.20(±2.24)/7.5mL

Kim 2019	Size based - Microfluidics	Immunocytochemical (IF) Cytomorphological analysis	EpCAM, CK	34	55.9	61.8	2.0 [1-6]/5mL
Naoe 2019	Size based - Microfluidics	Flow cytometry IF	CA9	13	38.5	69.2	2/4mL*
Wu 2019	Antibody based - IM(-)	FISH, Immunocytochemical (IF)	CEP8, CK18	8	0	75.0	5.5 [0-12]/7.5mL
Haga 2020	Antibody based - IM(-)	Flow cytometry IF	EpCAM, CK	54	8.3	100.0	LRN 3.4±4.2/4mL ORN 7.7±6.8/4mL LPN 3.4±4.1/4mL OPN 6.0±7.6/4mL
Emamekhoo 2020†	Antibody based - IM(+)	Immunocytochemical (IF)	CA9, CA12, CK, CD45/34/66b	26	100	100	CK+ 5 [0-53/mL] CA12+ 1 [1-9]/mL CA12+/CK+ 7 [0-102]/mL

CEP8 = chromosome 8 centromere probe; **CG =** chromogenic; **CK =** cytokeratin; **CSS =** cancer specific survival; **CTCn =** circulating tumor cell count; **DGC =** density gradient centrifugation; **DR =** detection rate; **EpCAM =** epithelial cell adhesion molecule; **FISH=** fluorescence in-situ hybridization; **IF =** immunofluorescence; **IM(+) =** immunomagnetic positive enrichment; **IM(-) =** immunomagnetic negative enrichment; **LPN =** laparoscopic partial nephrectomy; **LRN =** laparoscopic radical nephrectomy; **MB =** membrane based; **NA =** not applicable; **OA =** overall; **OPN =** open partial nephrectomy; **ORN =** open radical nephrectomy; **OS =** overall survival; **PFS =** progression-free survival.

† Grey Literature; * Calculated median based on data provided

3.4.2. CTC detection methods

After sample enrichment, CTC detection is performed by one of five different techniques: immunocytochemistry (32 articles), reverse transcriptase polymerase chain reaction (RT-PCR) (13 articles), cytomorphological criteria (11 articles), flow cytometry immunofluorescence (5 articles) and fluorescence in situ hybridization (FISH) (4 articles) (Figure 2).

3.4.2.1. Immunocytochemical analysis — Chromogenic or immunofluorescence

Most research groups use immunocytochemistry for CTC detection, which can be performed in one of two ways. The first is chromogenic immunocytochemistry, which uses enzyme-coupled antibodies to catalyze subtracts into an insoluble and chromogenic precipitate visible on standard light microscopy.[11,25–27,54,57,62] The second is immunofluorescence, which uses antibodies coupled to a fluorophore for visualization on fluorescence microscopy.[18,28,30–39,49,52,53,59,63–67] Molecular markers used vary widely, being CKs the most common (Table 1).

3.4.2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Most initial works used RT-PCR for CTC detection. This technique does not detect actual CTCs, but rather infers their presence by detecting circulating RNA of specific RCC markers, such as CK19[40], carbonic anhydrase (CA) 9[41,42,44,45,48,68], Von Hippel Lindau (VHL) gene mutation[43], prostate-specific membrane antigen (PSMA)[44], cadherin 6[6,46,47], CK8[69], or vimentin.[69]

3.4.2.3. Cytomorphological criteria on microscopy

Many authors rely on cytomorphological criteria for CTC identification, most often as a complementary analysis of immunocytochemistry. [11,18,26,27,38,51,54,56,58,59] Although not unanimous, the usual criteria for CTC definition are 1) large cell size, 2) large nuclear size with high nuclear/cytoplasmic ratio, 3) irregular nuclear membrane, and 4) cell irregularity.

3.4.2.4. Flow cytometry immunofluorescence

Flow cytometry immunofluorescence has recently been reported as a technique allowing faster, cheaper, and more automated sample processing.[60,70–73]

3.4.2.5. Fluorescence in situ hybridization (FISH)

FISH probes targeting VHL gene and chromosome 8 aneuploidy have been used by some groups, [61,74,75] with one group using RNA FISH for several markers.[10]

3.4.3. CTC performance as a biomarker

CTCs are being studied as a diagnostic (Table 1), prognostic (Table 2), and treatment response (Table 3) biomarker.

3.4.3.1. Diagnostic biomarker

3.4.3.1.1. CTC detection rate (CTC DR)

CTC DR varied widely between techniques, molecular markers, and research groups, ranging from 10 to 100%, with a median of 57% (interquartile range [IQR] 55).

Research groups using MACS followed by immunocytochemistry reported similar DRs, ranging from 32.2 to 41.7%.[11,25–27] On the other hand, studies conducted with CellSearch® showed DRs ranging from 16.0 to 92.0%.[32–38,69] In the only study comparing techniques, ISET® showed a 36.1% and CellSearch® a 19.4% DR, with low consistency between both (Kappa=0.063, p=0.673).[38] Liu S. et al. was the only group so far to target CA9 and CD147 using an antibody-based microfluidics chip, achieving a 94.7% DR.[18] By contrast, DR using EpCAM was only 18.6%. More recently, Haga et al.[70], as well as some gray literature[71,72], obtained 100% CTC DR using flow cytometry immunofluorescence.

Most groups used peripheral blood samples, but renal vein blood was also studied.[40,41,43,45] Uemura et al. found a higher DR in renal vein (33.3%) than in peripheral blood (18.5%).[45] Conversely, Hioki et al. reported a 32% DR in peripheral blood compared to 25% in the renal vein.[40]

Several studies found a positive correlation between DR and tumor staging.[11,27,38,40,46,51,59] Hioki et al. reported a 0% DR for stage 1, 25% for

stage 2, 75% for stage 3, and 100% for stage 4 tumors (p=0.002).[40] Shimazui et al. showed concordant results, with 70.4% in M1 and 45.0% in M0 patients (p=0.037).[46] Blümke et al. reported a significant correlation with N and M status, with 42% DR for N0, 86% for N1, 91% for N2 (p<0.001), 43% for M0, and 67% for M1 patients (p=0.014).[11,27]

3.4.3.1.2. CTC count (CTCn)

Thirty-three articles reporting CTCn were identified. Some showed an association between CTCn and clinicopathological features. Theil et al. found a significantly higher CTCn in patients with metastatic disease compared to localized disease, of 9.6 vs. 5.3 CTC/7.5mL, respectively.[62] Liu S. et al. reported a 2.2-fold increase in CTCn between late-stage (3 and 4) and early-stage (1 and 2) disease (p<0.001). This group additionally found higher vimentin-positive mesenchymal-type cell counts with increasing stage (p<0.001).[18] Haga et al. also showed higher preoperative CTCn in stage 4 disease compared to other stages, both on univariable (stage 1: 3.3±4.1, stage 2: 4.7±4.2, stage 3: 4.7±2.9, stage 4: 13.0±10.8, p<0.001) and multivariable (p<0.001) analysis, but not in postoperative counts.[70] On the other hand, several studies failed to show this correlation.[10,32,52,68] No correlation between RENAL score, Fuhrman grade, or venous invasion and CTCn was observed, both pre and post-operatively.[70]

3.4.3.2. Prognostic biomarker

Table 2 summarizes six studies that correlated CTC detection with prognostic outcomes. Four studies found a correlation between either CTC DR or CTCn and prognostic outcome measures.[27,28,37,48] Conversely, two studies showed no statistically significant correlation with survival outcomes.[38,59]

3.4.3.3. Treatment response biomarker

Seven studies measured CTCs in the perioperative setting (Table 3). Ashida et al. were the first to show an increased CTC DR after surgical manipulation: 11.8% before surgery, 37.5% on D1, 18.8% on D7, and 18.2% on D30.[43] Similarly, El-Heliebi et al. reported a significant increase in CTC DR in the immediate postoperative period, from 29% pre-op to 40% on D1 (p=0.04), decreasing to 33%

on D8.[54] Interestingly, two studies revealed a 100% DR, both before and after surgery for localized disease.[10,70]

Haga et al. found a higher immediate post-op CTCn after open radical nephrectomy (ORN), with an increase from 7.7±6.8 to 22.5±26.3 ($p<0.05$), but not after laparoscopic radical nephrectomy (LRN), open partial nephrectomy (OPN), or laparoscopic partial nephrectomy (LPN).[70]

Wang et al. studied CTCn kinetics in patients with localized disease at 6 and 12 months post-op.[10] Patients were stratified according to metastases development on follow-up. In the M1 group, a progressive increase in CTCn was observed, from 4.42±2.35 to 6.33±3.03 at 6 months and 9.8±5.03 at 12 months ($p<0.05$). In the M0 group, a progressive decrease in CTCn was reported, from 5.20±2.24 to 4.52±2.30 at 6 months and 3.63±1.37 at 12 months ($p<0.05$).

Only two studies compared CTC detection according to surgical technique and approach. Ohlman et al. found no difference in CTC DR between transperitoneal and retroperitoneal ORN.[68] Haga et al. found a significant increase in postoperative CTCn only in ORN but not in LRN, OPN, or LPN (LRN 4.8 ± 3.7, ORN 22.5 ± 26.3, LPN 7.9 ± 9.1, OPN 6.4 ± 6.3, $p<0.001$ on multivariable analysis). Additionally, the authors found that tumor diameter significantly correlated with post-op CTCn ($r=0.30$, $p=0.01$) but not pre-op CTCn. However, TNM staging does not seem to impact CTC release with surgery.[70]

Table 2. Studies assessing the correlation between CTCs and prognosis.

Reference (author year)	Prognostic Outcome Measure	Prognostic Outcome and Measure Effect Size
Gilbert 2006	5y DFS (CA9 + vs. -)	**39.5% vs. 88.1%, p=0.048**
	5y CSS (CA9 +vs. -)	85.7% vs. 96.0 %, p=0.57
	5y OS (CA9 + vs. -)	76.2% vs. 72.2%, p=0.93
	(median FU 4.7y)	
Bluemke 2009	OS (CK+ vs. CK- before surgery)	**RR 2.7, p=0.049**
	OS (CK+ vs. CK- after surgery)	**RR 4.3, p=0.036**
	OS (CK+ vs. CK- before and after surgery)	**RR 2.3, p=0.048**
	(median FU 15 mo)	
Nel 2016	PFS (n-cadherin+ CTCn <0.35 vs. 0.35/1000 PBMNC);	**7 vs. 15 months, HR 0.31 (CI 0.06-1.59), p=0.04**
	PFS (n-cadherin+ CTC vs. no CTC)	
	(median FU not stated)	
Basso 2017	PFS (CTC+ vs. CTC -)	**8.8 vs. 16.6 mo, HR 1.41 (CI 1.02-1.9), p = 0.03**
	PFS (CTC>=3 vs. CTC <3/7.5mL)	**5.8 vs. 15 mo, HR 1.99 (CI 1.28-3.03), p = 0.002**
	OS (CTC>=3 vs. CTC <3/7.5mL)	**13.8 vs. 52.8 mo, HR 1.99 (CI 1.17-3.2), p = 0.003**
	(median FU 31.5 mo)	
Bai 2018	OS (CTC+ vs. CTC-) CellSearch	HR 1.78 (CI 0.48-6.57), p = 0.391
	OS (CTC+ vs. CTC-) ISET	HR 0.57 (CI 0.18-1.78), p = 0.336
	PFS (CTC+ vs. CTC-) CellSearch	HR 1.43 (CI 0.4-5.15), p = 0.581
	PFS (CTC+ vs. CTC-) ISET	HR 1.95 (CI 0.68-5.6), p = 0.214
	(median FU 36 mo)	

Kim 2019	2y PFS (CTC + vs. -)	81.0% vs. 100.0%, p=0.704
	2y CSS (CTC + vs. -)	38.5% vs. 49.2%, p=0.158
	(median FU 19.5 mo)	

CI = confidence interval; **CK =** cytokeratin; **CSS =** cancer-specific survival; **CTC =** circulating tumor cell; **DFS =** disease-free survival; **FU** = follow up **HR =** hazard ratio; **mo =** months; **OS =** overall survival**; PBMNC =** peripheral blood mononuclear cells; **PFS =** progression-free survival; **RR =** relative risk; **y =** years.

Highlighted words mean statistically significant differences.

Table 3. Studies assessing CTCs in the perioperative setting.

Reference (author/year)	Patients (n)	Pre-op CTC DR (%)	Post-op CTC DR (%)	Pre-op CTCn (median or mean, units)	Post-op CTCn (median, [range], units)
Ashida 2000	20	11.8	D1 37.5 D7 18.8 D30 18.2	—	—
Shimazui 2003	87	52.9	D7-21 54.2	—	—
Ohlman 2006	24	45.8	58.3	—	—
Bluemke 2009	154	37.1	D5-7 45.4	6/16mL (range 1-51)	—
El-Heliebi 2013	30	29	D1 40.0 D8 33.0	3/8mL (range 1-62)	D1 3 [1-37]/8mL D8 3 [1-58]/8mL
Wang 2019	69	100	6-12M 100.0	Overall: Epithelial 8.21 (±4.77)/7.5mL Mesenchymal 2.30 (±1.41)/7.5mL Mixed 5.20 (±2.24)/7.5mL	6M (M0 on FU): Epithelial 1.39 (±0.84)/7.5mL Mesenchymal 2.02 (±1.58)/7.5mL Mixed 4.52 (±2.30)/7.5mL
				M0 on FU: Epithelial 0.91 (±0.57)/7.5mL Mesenchymal 2.30 (±1.41)/7.5mL Mixed 5.20 (±2.24)/7.5mL	6M (M1 on FU): Epithelial 2.66 (±1.78)/7.5mL Mesenchymal 2.66 (±1.30)/7.5mL Mixed 6.33 (±3.03)/7.5mL
				M1 on FU: Epithelial 0.85 (±0.46)/7.5mL Mesenchymal 2.42 (±1.40)/7.5mL Mixed 4.42 (±2.35)/7.5mL	12M (M0 on FU): Epithelial 0.72 (±0.33)/7.5mL Mesenchymal 2.47 (±1.98)/7.5mL **Mixed 3.63 (±1.37)/7.5mL**
					12M (M1 on FU): Epithelial 2.20 (±1.83)/7.5mL

						Mesenchymal 4.60 (±2.39)/7.5mL **Mixed 9.80 (±5.03)/7.5mL**
Haga 2020	54	100	D0 100.0	LRN 3.4 ± 4.2/4mL ORN 7.7 ± 6.8/4mL LPN 3.4 ± 4.1/4mL OPN 6.0 ± 7.6/4mL	Immediate post-op:	LRN 4.8 ± 3.7/4mL **ORN 22.5 ± 26.3/4mL** LPN 7.9 ± 9.1/4mL OPN 6.4 ± 6.3/4mL

CTCn = circulating tumor cell count; **DR =** detection rate; **FU =** follow-up; **LPN =** laparoscopic partial nephrectomy; **LRN =** laparoscopic radical nephrectomy; **OPN =** open partial nephrectomy; **ORN =** open radical nephrectomy.

Highlighted words mean statistically significant differences.

3.5. DISCUSSION

CTCs have the potential to improve our understanding of cancer biology and to become a relevant diagnostic, prognostic, and treatment-monitoring biomarker. Liquid biopsies can be repeated to provide real-time information on disease status and molecular profile. However, there is currently not enough evidence to recommend CTC detection in clinical practice in RCC.

This review identified studies which revealed many limitations, generally with a high risk of bias and a low level of evidence. There are no prospective randomized trials to date and most studies are exploratory in nature. Most designs are observational descriptive and only a few cohort studies have been published, with small and unbalanced samples. Blood sample collection site, timing, number, and volume differed widely. Furthermore, enrichment and detection techniques, as well as targeted molecular markers varied considerably, hindering comparisons between studies. Hence, no robust conclusions can be drawn. In this review, we chose to include gray literature due to the exploratory phase of this research field. This may be regarded both as an advantage, allowing inclusion of emerging methods which have not yet been published, and a limitation, since methods and results are frequently interim and cannot be thoroughly analyzed.

Results of CTC DR varied widely between studies and were not consistent between technical platforms and molecular markers. Divergence in results is not fully explained by differences in clinical characteristics and may be associated with the complexity of CTC multi-step processing techniques, causing cell loss and limiting sensitivity.[76] Additionally, tumor multi-clonality and EMT can change the expression of molecular markers, both in primary tumors and CTCs.[77–79] These phenomena are frequent in RCC and may lead to further CTC subsets loss. To overcome these limitations, the combination of different markers has been attempted, including mesenchymal markers like vimentin.

Being the only FDA-approved technology for CTC detection, CellSearch® is considered the reference standard in most epithelial cancers, but not in RCC.[16] Initial studies targeting EpCAM showed particularly low DR.[25–27,33,34,39] In fact, not all types of CTCs express EpCAM[80,81] and it has been reported that only 18.6% of RCC CTCs express EpCAM[18]. However, recent studies found a 100% DR with EpCAM and/or CK using flow cytometry immunofluorescence or size-based

enrichment followed by RNA FISH.[10,70–72] This points to the importance of optimizing both molecular markers and enrichment and detection techniques to maximize performance.

Both CTC presence and CTCn have been shown to correlate with staging, particularly N+ and M+ status.[11,18,27,38,40,46,51,59,62,70] Vimentin-expressing CTCs also seem to correlate with more advanced tumor stage in RCC.[18] However, the role of CTCs as a diagnostic biomarker is still uncertain. It would be interesting to study if CTC presence, quantification, and characterization can be used for RCC risk stratification.

Only six papers correlated CTCs with prognostic outcome measures, with conflicting results. These studies had short follow-ups and outcome measures were not primary endpoints. Although CTC presence was significantly associated with lower OS in a study by Bluemke et al.[27], two other studies did not confirm this finding.[38,48] Concurrently, the impact of CTC detection in PFS was demonstrated in only two of four studies.[28,37] In the single study correlating CTCn with prognostic outcomes, Basso et al. found it had an inverse correlation with OS.[37] Further studies designed to address the prognostic impact of CTCs are necessary before they can become a part of clinical decision pathways.

A correlation between surgical manipulation and CTC release has previously been demonstrated. In colon cancer, a no-touch isolation technique reduces intraoperative shedding of tumor cells into the portal vein during resection.[80] In genitourinary cancers, CTC increase after surgery has also been observed in transurethral resection of the bladder (TURB) and brachytherapy patients.[81,82] In this review, four studies found a CTC increase after surgery,[27,43,54,68] while another study found no difference.[46] Two research groups investigated post-op CTC DR kinetics and both found a CTCn rise in post-op D1 followed by a decrease after one week, but still above the pre-op value.[43,54] Interestingly, however, two studies revealed CTCs in all patients, both before and after surgery for localized disease.[10,70] These findings suggest that with a sufficiently sensitive detection method, all patients will present CTCs, which may render CTC DR clinically obsolete. This points towards CTCn as the true biomarker to consider for clinical use. However, CTC persistence in patients who remain relapse-free after radical surgery is still to be explained. Only Wang et al. studied CTC kinetics on longer follow-up. Patients who developed metastases showed an increase in CTCn, unlike

patients who remained metastases-free, who showed a progressive decrease in CTCn[10]. CTCs may thus be a promising surrogate marker for micro metastases, similarly to serum biomarkers for testicular cancer. Studies with larger samples and longer follow-up are required to test this hypothesis.

Haga et al. were the only to compare CTC release among different surgical approaches[70]. Only ORN showed increased post-op CTCn, unlike LPN, OPN, and LRN on multivariable analysis, although this group had a very significant selection bias towards increased TNM. Properly designed prospective comparative studies are needed to answer the question of whether surgical approach and no-touch protocols can reduce CTC release.

A clinical RCC biomarker remains elusive. CTCs could be such a biomarker, but so far the lack of robust evidence and technical standardization restricts its clinical use. A sensitive, specific, reproducible, and cost-effective detection technique which can be approved by regulatory agencies is still under investigation.

Further research efforts should focus on improving CTC detection accuracy, standardizing CTC definitions and detection methods, and comparing platform performance. Appropriate study designs, with adequate follow-up and clinical outcomes, are required to incorporate these liquid biopsies in clinical decision algorithms for RCC management.

3.6. CONCLUSIONS

CTCs have the potential to be a diagnostic, prognostic, and treatment monitoring tool in the patient-tailored management era. CTC research in RCC is still in an exploratory phase and no standard detection technique or molecular markers are available. Well-designed prospective studies with standardized methodology are required to bring liquid biopsy into clinical practice.

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3.8. SUPPLEMENTARY MATERIAL

Table 4.1. Risk of bias assessment — Newcastle-Ottawa Quality Assessment Scale.

Reference (first author/year)	Score (Reviewer 1)				Score (Reviewer 2)				Score (Reviewer Cosensus)			
	S	C	O/E	Total	S	C	O/E	Total	S	C	O/E	Total
Shimazui 2004	**	-	**	4/9	***	-	**	5/9	****	-	***	7/9
Ohlman 2006	***	-	**	5/9	***	-	**	5/9	***	-	**	5/9
Bluemke 2009	**	-	*	3/9	***	**	*	6/9	***	**	*	6/9
Rossi 2012	***	-	*	4/9	***	-	*	4/9	***	-	*	4/9
Bai 2018	***	-	*	4/9	***	-	***	6/9	***	-	***	6/9
Kim 2019	***	*	**	6/9	***	-	***	6/9	***	-	***	6/9
Wang 2019	***	-	**	5/9	**	-	***	5/9	**	-	***	5/9
Haga 2020	***	-	*	4/9	***	-	*	4/9	***	-	*	4/9
Basso 2017	***	-	**	5/9	***	-	**	5/9	***	-	**	5/9

S = Selection; C = Comparability; O/E = Outcome/Exposure.

Table 4.2. Risk of bias assessment — JBI Critical Appraisal Checklist for Case Series.

Reference (first author/year)	Reviewer 1				Reviewer 2				Reviewer Consensus			
	Yes	No	Unclear	Total*	Yes	No	Unclear	Total*	Yes	No	Unclear	Total*
Glaves 1988	2	5	2	9	2	4	4	10	2	4	3	9
Ashida 2000	5	2	2	9	5	4	1	10	4	2	3	9
Bilkenroth 2001	3	3	3	9	4	4	2	10	3	3	3	9
Meye 2002	5	2	2	9	4	4	2	10	5	1	3	9
Uemura 2003	3	4	2	9	2	5	3	10	2	4	3	9
Shimazui 2003	3	5	1	9	5	2	2	9	5	2	2	9
Bluemke 2005	2	5	2	9	1	7	2	10	2	4	3	9
Gradilone 2011	2	5	2	9	2	4	4	10	3	3	3	9
El-Heliebi 2013	7	1	1	9	7	1	1	9	7	1	1	9
Liu S. 2016	6	2	1	9	8	1	1	10	6	2	1	9
Nel 2016	5	3	1	9	5	5	0	10	6	2	1	9
Liu M. 2017	7	1	1	9	9	1	0	10	7	1	1	9
de la Taille 2000	4	4	1	9	4	3	3	10	4	3	2	9
Li 2005	5	3	1	9	4	5	0	10	5	2	2	9
Xing 2018	3	3	3	9	4	5	1	10	3	3	3	9
Williams 2012	2	4	3	9	1	1	8	10	2	2	5	9
Yip 2014	4	3	2	9	2	1	7	10	4	1	4	9
Mittal 2014	4	2	3	9	6	0	4	10	5	0	4	9
Lomo 2006	2	0	7	9	1	1	8	10	2	1	6	9
Emamekhoo 2020	2	4	3	9	6	0	4	10	4	2	3	9
Fehm 2002	2	5	2	9	3	4	3	10	2	4	3	9
Kang 2018	2	5	2	9	2	4	4	10	2	4	3	9
Zhenlong 2018	3	3	3	9	3	4	2	9	4	4	1	9
Gilbert 2006	7	2	1	10	7	0	3	10	6	1	2	9
Hioki 1999	4	2	4	10	5	1	4	10	4	2	3	9
Mittal 2012	4	3	2	9	6	0	4	10	4	1	4	9
Gutschi 2010	2	6	1	9	2	1	7	10	2	1	6	9
Arafat 2017	2	4	3	9	1	1	8	10	2	1	6	9
Naoe 2019	4	2	3	9	3	4	3	10	4	2	3	9
Zhang 2016	5	3	2	10	8	2	0	10	6	0	3	9
Gorin 2015	2	5	2	9	1	1	8	10	2	4	3	9

Novikova 2015	2	5	2	9	2	1	7	10	2	1	6	9
Iacovelli 2011	2	4	3	9	2	1	7	10	2	1	6	9
Desotelle 2016	1	5	3	9	1	2	7	10	1	2	6	9
Uemura 1999	2	6	2	10	3	6	1	10	3	4	2	9
Wu 2019	4	3	2	9	3	4	3	10	4	3	2	9
Amato 2015	2	4	3	9	2	1	7	10	2	1	6	9
Seideman 2009	4	4	2	10	3	1	6	10	4	1	4	9
Broncy 2018	6	1	2	9	6	0	4	10	6	0	3	9
Ionescu-Zanetti 2015	1	5	3	9	2	1	7	10	2	4	3	9
Tseng 2015	2	4	3	9	2	1	7	10	2	4	3	9
Ramirez 2012	2	4	3	9	2	1	7	10	2	1	6	9
McKiernan 1999	6	3	1	10	6	4	0	10	6	1	2	9
Allard 2004	2	6	1	9	1	7	2	10	2	4	3	9
Theil 2014	2	4	3	9	2	1	7	10	2	4	3	9

*Total applicable

SEARCH STRINGS

Embase <1974 to 2020 April 01>, Ovid MEDLINE(R) and Epub Ahead of Print, In-Process & Other Non-Indexed Citations, Daily and Versions(R) <1946 to April 01, 2020>

Search Strategy:

1 ((Kidney or renal) adj3 (cancer* or tumo?r or neoplasm* or carcinoma* or neoplasia*)).tw. (141612)

2 "Renal cell carcinoma*".tw. (83363)

3 grawitz tumo?r.tw. (158)

4 "collecting duct carcinoma*".tw. (811)

5 renal cell carcinoma/ (37869)

6 1 or 2 or 3 or 4 or 5 (147039)

7 "circulating tumo?r cell*".tw. (15716)

8 "circulating neoplastic cell*".tw. (88)

9 "embolic tumor cell*".tw. (28)

10 CTC.tw. (17811)

11 tumor embolism/ (12663)

12 circulating tumor cell/ (18969)

13 7 or 8 or 9 or 10 or 11 or 12 (36808)

14 6 and 13 (1206)

15 remove duplicates from 14 (954)

Web of Science:

ALL=(kidney cancer) OR ALL=(renal cancer) OR ALL=(kidney tumor) OR ALL=(renal tumor) OR ALL=(kidney tumour) OR ALL=(renal tumour) OR ALL=(renal cell carcinoma) OR ALL=(renal cell cancer) OR ALL=(grawitz tumor) OR ALL=(grawitz tumour) OR ALL=(collecting duct carcinoma) OR ALL=(clear cell renal carcinoma) OR ALL=(collecting duct carcinoma)

AND

ALL=(circulating tumor cell*) OR ALL=(circulating tumour cell*) OR ALL=(circulating neoplastic cell*) OR ALL=(embolic tumor cell*)

Search on April 1, 2020

4. RESEARCH ARTICLE: Clinical Validation of a Size-Based microfluidic device for circulating tumor cell isolation and analysis in renal cell carcinoma

4. TITLE: Clinical validation of a size-based microfluidic device for circulating tumour cell isolation and analysis in renal cell carcinoma patients

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4.1. ABSTRACT

Renal Cell Carcinoma (RCC) presents as a metastatic disease in a third of the cases. Liquid biopsy research on circulating tumor cells (CTCs) is improving the understanding of RCC biology and metastasis formation. There is a need for a standardized, sensitive, specific, and cost-effective CTC detection technique. Platforms relying solely on epithelial markers are inappropriate in RCC due to the frequent epithelial-to-mesenchymal transition (EMT) that CTCs undergo. We aim to test and clinically validate the RUBYchip™, a microfluidics label-free CTC detection platform, in RCC patients. The average capture efficiency was 74.9% in spiking experiments using three different RCC cell lines. Clinical validation was done in a cohort of 18 patients, with an average CTC detection rate of 77.8%. The average total CTC count was 6.4 (0-27), 101.8 (0-255), and 3.2 (0-10), and the average of mesenchymal CTCs (single and in clusters) was zero, 97.6 (0-255), and 0.2 (0-1), for M0, M1 treatment-naive (M1TN), and M1 progressing-under-treatment (M1TP) groups, respectively. CTC clusters were detected in 25% and 60% in M0 and M1TN patients, respectively. In conclusion, our results demonstrate that RUBYchip™ is an effective and reliable CTC detection platform in RCC.

KEYWORDS: kidney cancer; renal cell carcinoma; circulating tumor cells; liquid biopsy; microfluidics

4.2. INTRODUCTION

Kidney Cancer (KC) is the 14th most common malignancy worldwide, with a global incidence of 431,288 in 2020 [1]. The incidence in Europe and North America is considerably higher than in other regions, ranging from 2.09 cases per 100,000 inhabitants (age-standardized rate) in Middle Africa to 24.7 in North America [2]. Renal cell carcinoma (RCC) accounts for the great majority (90%) of KC cases [2]. The predominant histological subtypes are clear cell RCC (ccRCC) (70%), papillary RCC (pRCC) (10–15%), and chromophobe RCC (5%) [2]. The other histological subtypes account for less than 1% each [2]. About a third of RCC patients are diagnosed at a metastatic stage and up to 40% of patients treated with curative intent relapse and develop metastasis during follow-up [3,4].

A clinically useful biomarker is missing for RCC. Diagnosis and follow-up are still solely dependent on cross-sectional imaging. Several biomarkers have been studied, but none have yet reached the needed accuracy and ease of use for clinical application, particularly in guiding disease management.

The main focus of current research in the search for an RCC biomarker is liquid biopsies. The principle of liquid biopsies is to obtain tumor-derived biological material circulating in the bloodstream, with a simple blood sample. The objective is to access phenotypic and genetic information about the primary and secondary tumors, without the invasion of a tumor or metastasis biopsy. This can allow minimally invasive early cancer diagnosis, and repeated sequential sampling during disease management, in order to accurately guide treatment decisions, monitor treatment response, and provide prognostic information. Liquid biopsy can focus on a multitude of circulating biomarkers, such as circulating tumor DNA (ctDNA), micro RNA (miRNA), and circulating tumor cells (CTCs) [5–8].

In liquid biopsy research, CTCs have been the focus of particular interest. The potential clinical value of CTCs has been explored in several tumor types, like breast, colon, and prostate cancer [9]. Five studies found a correlation between CTC presence and counts and prognostic outcome measures in RCC, despite having short follow-ups and the fact that these outcome measures were not

primary endpoints [10–14]. Vimentin-expressing CTCs also seem to correlate with more advanced RCC stages [15].

The scientific community is still searching for a sensitive, specific, reproducible, and cost-effective CTC detection technique. CTCs are extremely rare compared to whole blood cells, estimated as one CTC per billion normal blood cells in metastatic cancer patients [16]. CTC enrichment techniques can be classified into four categories: antibody-based (immunomagnetic beads or microfluidics), density-based, size-based (microfluidics and membrane filters), and electrophoresis-based [4]. Five different techniques have been used for CTC detection and identification: immunocytochemistry, reverse transcriptase polymerase chain reaction (RT-PCR), cytomorphological criteria, flow cytometry with immunofluorescence, and fluorescence in-situ hybridization (FISH) [4]. Cellsearch® is the only FDA-approved CTC detection platform for clinical use. It uses immunomagnetic enrichment and fluorescent labeling for CTC detection [17]. However, it is deemed inappropriate for RCC due to the lack of epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) expression in many CTCs that have undergone epithelial-to-mesenchymal transition (EMT), a very common phenomenon in this type of cancer [15]. Only 18.6% of RCC CTCs express EpCAM [15]. This is why initial studies using this marker showed particularly low CTC detection rates [18,19].

The isolation of CTCs based on their physical properties is a simpler albeit more efficient method, independent of molecular markers, focusing on differences in cell size, and deformability. Nonetheless, although more sensitive, it may have less specificity, given CTC heterogeneity [20]. Size-based CTC isolation platforms are based on greater size and decreased deformability of CTCs compared to blood cells [4]. They have the advantage of minimizing cell loss and allowing downstream analysis of intact cells. Their main limitations are device clogging and some loss of CTCs which are smaller than the pore of the device. Several authors have used size-based CTC isolation in RCC patients, both membrane-based [21–28] and microfluidic devices [29–31]. Most of them are designed to capture cells bigger than 8 μm, letting both smaller erythrocytes and the deformable leukocytes pass through [32]. Microfluidic-based devices minimize sample

processing steps, needing shorter processing time since no sample pre-processing is required [33]. They also require reduced reagent volumes and have low contamination issues and sample loss rates. Hence, cell loss is minimized, especially in samples with low CTC concentration, resulting in a superior sensitivity and detection rate [4].

Considering their potential advantages, microfluidic devices have been investigated as promising CTC isolation methods. RUBYchip™ proved significantly superior to the FDA-approved CellSearch® in the isolation of CTCs from breast, colorectal, gastric, and pancreatic cancers [34–36].

The objective of this study is to test and validate the RUBYchip™ microfluidic device for CTC isolation and analysis in RCC patients.

4.3. MATERIALS AND METHODS

4.3.1. Microfluidic Device

The RUBYchip™ device (RUBYnanomed / International Iberian Nanotechnology Laboratory (INL), PCT/EP2016/078406) is a microfluidic system that captures CTCs from whole blood samples based on cell size and deformability [36]. The device is constituted by an inlet that channels the sample through a series of interconnected capillaries into several cell-filtering chambers with transverse single rows of micropillars, comprising the cell-filtering area. The size, geometry, and gap size of the pillars were designed so that deformable white blood cells gently flow through, while larger, more rigid cells, like CTCs, are retained in the cell-filtering chamber. The fabrication process, technical specifications, and details of the device were previously described in a publication by Lopes C, *et al* [36].

4.3.2. Cell culture

Human kidney cancer cell lines Caki-2 (ATCC, HTB-47), A-498, and 786-O were used for spiking experiments. The Caki-2 cell line was cultured in McCoy's 5A Medium (Gibco™, 12330031), the A-498 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™, 41966029), and the 786-O cell line in Roswell Park Memorial

Institute (RPMI) 1640 (Gibco™, 21875034). All growth media were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco™, 10270106) and 1% Penicillin/Streptomycin (Gibco™, 15140122). All cell lines were maintained at 37ºC with 5% CO_2 in a humidified atmosphere, at a low passage, and routinely tested for mycoplasma contamination by qPCR (GATC Biotech).

4.3.3. Spiking experiments

The capture efficiency of the RUBYchip™ device in RCC was assessed through spiking experiments using three cell lines — Caki-2, A-498, and 786-O. Approximately 200 cells were labeled with DAPI (10 µg/mL, Sigma-Aldrich) after trypsinization and added to 7.5 mL of whole blood samples from healthy donors. To find the best conditions, samples were injected in the RUBYchip™ using a syringe pump (KF Technology) at three different flow rates — 80, 100, and 120 µL/min. Afterward, devices were washed with 2% Bovine Serum Albumin (BSA) (NZytech Lta) in PBS 1X, fixed with 4% formalin for 20 min at room temperature, and finally washed with 0.5% BSA in PBS 1X followed by 1% sodium azide (Sigma Aldrich) in PBS 1X. As previously described, cell counting control of the spiked cell number was performed by pipetting the same cell suspension volume into a well plate [36]. Fluorescence images of the cells in the devices and well plates were acquired using an inverted fluorescence Nikon Eclipse Ti microscope at 20x magnification. Capture efficiency was calculated as the ratio between the number of DAPI-positive cells trapped inside the device and the average cell count in the well plate as shown in equation 1, as previously described in Ribeiro-Samy S, *et al* [35]. Experiments were performed in triplicate.

$$CTC\ capture\ efficiency\ (\%) = \frac{Trapped\ cells}{Spiked\ cells} \times 100\ (\%)$$

Equation 1. Calculation of capture efficiency as the ratio between the number of DAPI-positive cells trapped inside the device and the average cell count in the well plate.

4.3.4. Immunocytochemistry protocol and immunofluorescence imaging

Different experimental conditions were tested to optimize the CTC staining protocol using the cells trapped in the spiking experiments as models. The

selected antibody panel included AF647 anti-human vimentin (Biolegend, 1:50), PE anti-human CD45 (Invitrogen, Thermo Fisher Scientific, 1:50), and DAPI (1 µg/mL). To stain cytokeratin, two antibodies were tested: anti-human cytokeratin 8/18 unconjugated ready-to-use antibody (Dako, 200 µL), detected with FITC goat anti-rabbit IgG Cross-Adsorbed Secondary Antibody (Invitrogen, Thermo Fisher Scientific, 1:1000); and as a second option a FITC conjugated anti-human cytokeratin. After isolation, cells were permeabilized with 0.25% Triton X-100 for 10 min, then rinsed with PBS 1X. The antibody panel was incubated for 1h at room temperature in the dark, after a blocking step with 2% BSA in PBS 1X, for 20 min. For the samples incubated with unconjugated cytokeratin antibody, incubation with the secondary antibody for 30 min at room temperature in the dark was followed. Washing was done with 0.5% BSA in PBS 1X and with 1% sodium azide in PBS 1X. Images from the devices were obtained using an inverted fluorescence Nikon Eclipse Ti microscope at 20x magnification.

4.3.5. Patient recruitment and sample collection

In order to validate the RUBYchip™ for clinical use in RCC, 18 patients were recruited at Centro Hospitalar Universitário Lisboa Norte (CHULN), Lisbon, Portugal, between August 2021 and May 2022. Patients were divided into three groups, eight with localized disease (M0 group) with samples collected prior to treatment with curative intent, and 10 with metastatic disease (M1 group), subdivided into a treatment-naive (M1TN) group and a progression-under-therapy (M1TP) group, where progression was defined according to RECIST criteria [37]. Objective tumor status was evaluated by the TNM criteria [38]. This study was approved by CHULN's Ethics Committee and was conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. All patients signed the informed consent form before sample collection. Single 7.5mL peripheral blood samples were collected in EDTA tubes, at the time of diagnosis in the M0 group, before systemic therapy in the M1TN group, and after tumor progression in follow-up cross-sectional imaging studies for the M1TP group. The samples were anonymized and a code was attributed before sample processing.

4.3.6. CTC isolation and characterization

Clinical samples were processed in the RUBYchip™ device within 40 min after blood collection. The 7.5mL blood samples were injected at 80 µL/min and the CTCs entrapped in the device were then washed and fixed, as described for the spiking experiments (section 2.3). The CTCs were stained inside the device with the antibody panel described before (section 2.4), and under the same conditions. The first 12 patients were stained with the anti-human unconjugated cytokeratin 8/18, and the last six patients were stained with the FITC conjugated anti-human cytokeratin.

After image acquisition, images were manually analyzed and cells were classified and enumerated in a randomized blind fashion, by two independent operators. No variability was found in the cytokeratin signal, using the two antibodies, but a reduction of the background FITC signal was observed with the conjugated antibody. Cells were identified as CTCs and discriminated according to their phenotype using the following criteria: epithelial CTCs were DAPI+/CD45-/CK+/Vim-, mesenchymal CTCs were DAPI+/CD45-/CK-/Vim+, and EMT CTCs were DAPI+/CD45-/CK+/Vim+ [35,36,39]. Concomitantly, to be classified as CTCs, cells had to present cell membrane integrity in brightfield, a round nucleus, and cell-like morphology. CTC clusters were defined as groups of two or more cells, as long as they comply with the above criteria. These groups of cells must have regular contours and be in close contact with each other to be considered a cluster [40].

4.3.7. Statistical analysis

Continuous variables were described using median, average, and range, and categorical variables were presented as absolute frequencies and percentages. Fisher's Exact Test was used to compare the three patient groups (M0, M1TN, and M1TP) concerning the presence/ absence of CTCs. Negative binomial regression was used to compare the average number of CTC counts between groups: M1TN vs. M1TP, and groups induced by presence vs. absence of lymph nodes, smoking habits, hypertension, diabetes, overweight/obesity, and antiplatelet therapy). Pearson's correlation between CTC counts and the quantitative clinical variables was calculated and tested. Overall survival (OS) was estimated using the

Kaplan-Meier estimator. Median OS and 95% confidence intervals (95% CIs) were computed. Results were considered statistically significant if the p-value was less than 0.05. Statistical analyses were performed with R Software v2022.07.1 (R Foundation for Statistical Computing, Vienna, Austria). Survival analysis was performed using GraphPad Prism 8 v8.4.3 (686).

4.4. RESULTS

4.4.1. CTC isolation efficiency

The spiking experiments performed with the RUBYchip™ showed a high cell retention/capture yield of RCC cells.

The overall optimized capture efficiency was obtained at a flow rate of 80 µL/min, enabling isolation of 77.7%, 77.2%, and 69.8% of spiked Caki-2, A-498, and 786-O, respectively (Figure 1A). The mean capture efficiency for all RCC cell lines tested was 74.9%. At 100 and 120 µL/min, capture efficiency was lower and with higher variability than at 80 µL/min. Considering these results, patient samples were subsequently processed at 80 µL/min, since it was considered the optimal flow rate.

4.4.2. Patient cohort characterization

Table 1 summarizes the clinicopathological characteristics of the patient cohort. The median age of diagnosis was 60 years old for M0 and M1TP groups and 71 years old for the M1TN group. Overall, eight patients (72.7%) had clear cell RCC, one patient (9.1%) had chromophobe RCC, and two patients (18.2%) had papillary RCC.

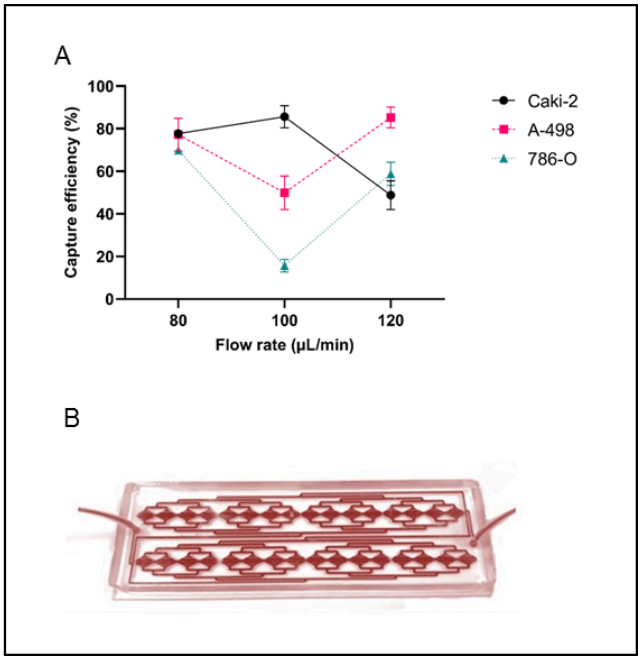

Figure 1. A. Capture efficiency (%) at three different flow rates (80, 100, and 120 µL/min) for Caki-2 (black circles with continuous line), A-498 (red squares with dashed line), and 786-O (blue triangles with dashed line) cells. For each flow rate, capture efficiency is represented as the mean and SD of triplicate experiments. **B. RUBYchip™ device running a blood sample.**

Table 1. Patients' clinicopathological characteristics

Clinicopathological characteristics	**Overall**	**M0**	**M1TN**	**M1TP**	**p-value**
Patient number	18	8	5	5	
Gender, n (%)					
Female	5 (28.0)	2 (25.0)	1 (20.0)	2 (40.0)	1
Age, years					
Median (range)	60 (43-78)	60 (52-70)	71 (43-78)	60 (48-69)	0.511
Smoking, n (%)	7 (38.9)	5 (62.5)	1 (20.0)	1 (20.0)	0.252
Obesity, n (%)					
Overweight/Obesity	12 (66.7)	7 (87.5)	1 (20.0)	4 (80.0)	0.05
BMI, score (%)					
Median (range)	25.5 (18-38.6)	25.5 (23.6-38.6)	21.5 (21.0-25.4)	25.5 (18.0-27.1)	0.049

Hypertension, n (%)	12 (66.7)	6 (75.0)	3 (60.0)	3 (60.0)	1
Diabetes, n (%)	5 (27.8)	3 (37.5)	1 (20.0)	1 (20.0)	1
ECOG, n (%)					0.384
0	10 (55.6)	4 (50.0)	3 (60.0)	3 (60.0)	
1	4 (22.2)	3 (37.5)	0	1 (20.0)	
2	2 (11.1)	1 (12.5)	0	1 (20.0)	
3	2 (11.1)	0	2 (40.0)	0	
T stage, n (%)					0.003
T1a	6 (33.3)	6 (75.0)	0	0	
T1b	3 (16.7)	2 (25.0)	1 (20.0)	0	
T2a	2 (11.1)	0	1 (20.0)	1 (20.0)	
T2b	2 (11.1)	0	1 (20.0)	1 (20.0)	
T3a	5 (27.8)	0	2 (40.0)	3 (60.0)	
N stage, n (%)					0.045
N0	13 (72.2)	8 (100.0)	3 (60.0)	2 (40.0)	
N1	5 (27.8)	0	2 (40.0)	3 (60.0)	
Histology, n (%)					0.515
Clear cell	8 (72.7)	4 (80.0)	1 (50.0)	3 (75.0)	
Chromophobe	1 (9.1)	0	1 (50.0)	0	
Papillary	2 (18.2)	1 (20.0)	0	1 (25.0)	
Metastasis, n(%)					1
Lung	-	-	3	3	
Bone	-	-	1	1	
Distant lymph nodes	-	-	1	1	
Antiplatelet therapy, n (%)	5 (27.8)	3 (37.5)	2 (40.0)	0	0.416
Systemic treatment, n (%)					
First line	-	-	2	2	
Second line	-	-	-	3	
Unfit for treatment	-	-	3	-	
Treatment, n (%)					
TKI	4 (22.2)	-	1 (20.0)	3 (75.0)	
ICI	3 (16.7)	-	1 (20.0)	2 (40.0)	

Radical nephrectomy	8 (72.7)	5 (62.5)	0	-
Partial nephrectomy	1 (9.1)	1 (20.0)	0	-
Surveillance	2 (11.1)	2 (25.0)	0	0
Not fit for treatment	3 (16.7)	0	3 (60.0)	0

M0 - localized disease patient group, M1TN - metastatic treatment-naive patient group, M1TP - metastatic progressing-under-treatment patient group, BMI - body mass index, ECOG - Eastern Cooperative Oncology Group score, TKI - tyrosine kinase inhibitors, ICI - immune checkpoint inhibitors; TNM according to the AJCC Cancer Staging Manual Eighth Edition, 2017 [38]. P-values concerning the comparison of the groups were obtained after Fisher's exact test for categorical variables and the Kruskal-Wallis test for the quantitative variables.

4.4.3. CTCs enumeration and characterization in RCC patients

Circulating tumor cells were found either as single CTCs or in clusters of two or more cells (Figure 2). The total CTC number was considered the sum of the number of single CTCs and the number of CTCs in clusters. Two CTC phenotypes were found in all patient groups, epithelial and mesenchymal CTCs. Of note, no EMT CTCs were detected in this cohort.

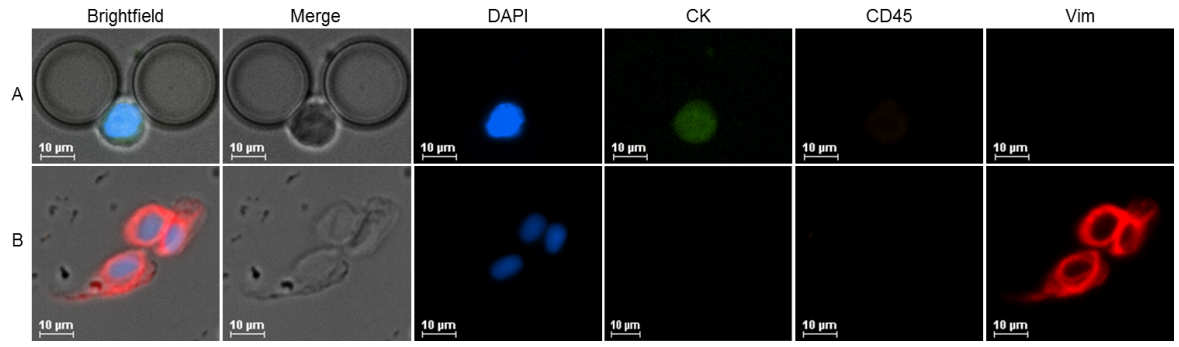

Figure 2. Representative fluorescence microscopy images of CTCs from RCC patients captured in the RUBYchip™.

A. Epithelial CTC (DAPI+/CD45-/CK+/Vim-). **B.** CTC cluster formed by mesenchymal CTCs (DAPI+/CD45-/CK-/Vim+). The images were acquired and observed with a 20X objective.

CTC counts stratified by phenotype and patient group (M0, M1TN, and M1TP) are presented in Table 2 and Figure 3. The overall detection rate was 77.8% (14/18),

75.0% in the M0 group (6/8), and 80.0% in the M1 group (8/10). CTC clusters were detected in 25.0% of M0 patients (2/8) and in 60.0% of M1TN (3/5) patients. Interestingly, in M1TP patients no CTC clusters were detected. All detected CTC clusters were composed of only mesenchymal cells.

Table 2. Median, average, and range of CTC counts and phenotype in the RCC patient's cohort. Total CTCs represent the sum of single CTCs and CTCs in clusters.

	M0			M1TN			M1TP		
	Median	Average	Range	Median	Average	Range	Median	Average	Range
Single CTCs	1.5	3.3	0-13	49	63.8	0-157	2	3.2	0-10
Epithelial	3	4.2	0-13	0	5.3	0-21	2	3	0-10
Mesenchymal	0	0.0	—	49	59.6	0-157	0	0.2	0-1
CTC Clusters	0	0.25	0-1	3	5.8	0-16	0	0.0	—
CTCs in clusters	0	3.1	0-21	31	38.0	0-98	0	0.0	—
Total CTCs	3	6.4	0-27	80	101.8	0-255	2	3.2	0-10
Epithelial	3	4.2	0-13	0	5.3	0-21	2	3	0-10
Mesenchymal	0	3.1	0-21	80	97.6	0-255	0	0.2	0-1

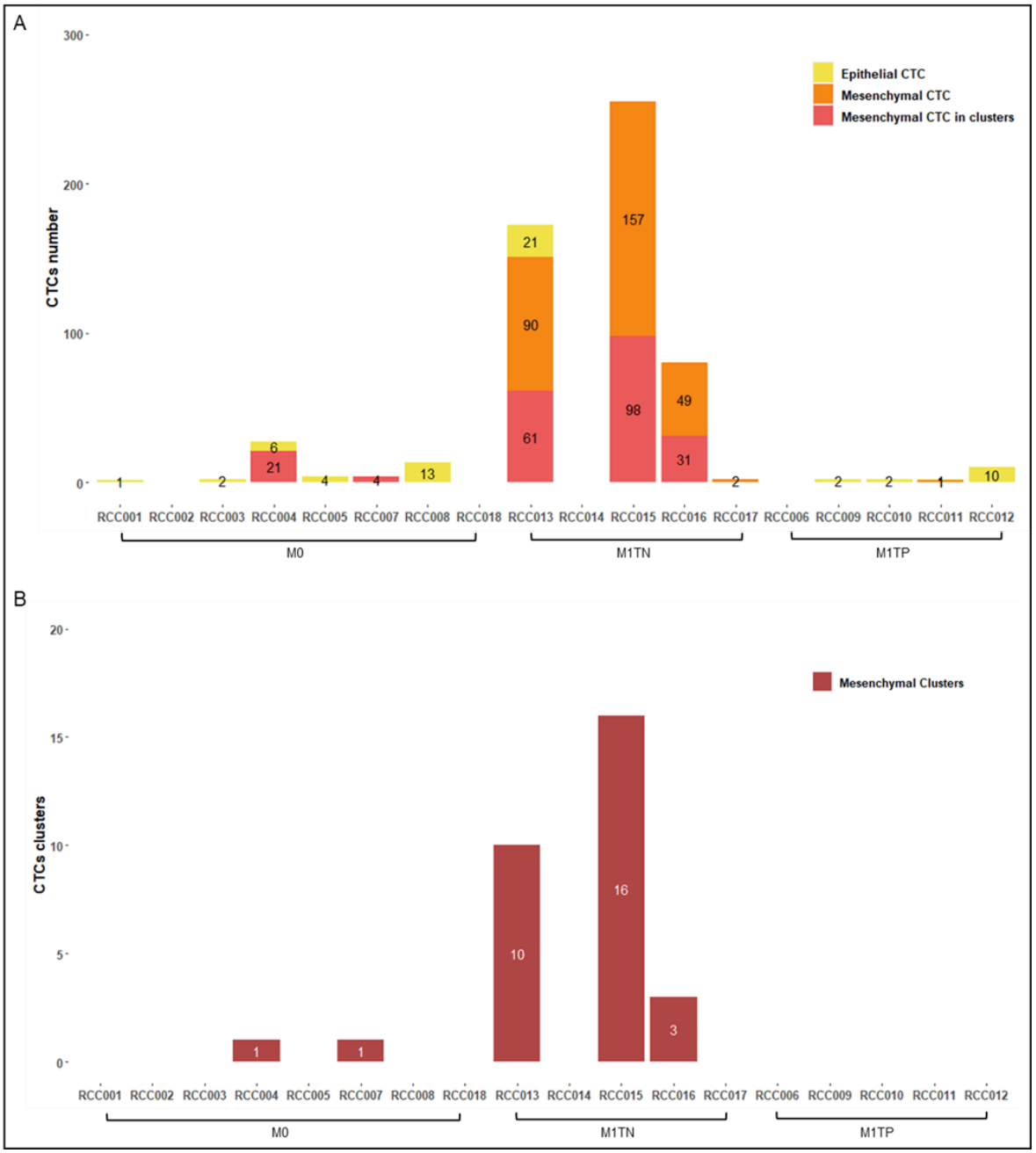

Figure 3. Graphics representing CTC numbers in each of the M0, M1TN, and M1TP patient groups.

A. Number and phenotype of single CTCs and CTCs in clusters. **B.** Number and phenotype of CTC clusters. Epithelial CTCs are represented in yellow bars, mesenchymal single CTCs in orange bars, mesenchymal CTCs in clusters as light red bars, and mesenchymal clusters in dark red bars.

The average total CTC count was 6.4 (0-27), 101.8 (0-255), and 3.2 (0-10), respectively for M0, M1TN, and M1TP groups. M1TN patients presented a

significantly higher number of CTCs than M1TP patients (31.8 times more on average, p=0.0003, 90% CI 30.0-345.6) (Figure 4). This difference is mainly due to the presence of mesenchymal CTCs. The average of total mesenchymal CTCs (single CTCs and CTCs in clusters) was 3.1, 97.6 (0-255), and 0.2 (0-1), respectively for M0, M1TN, and M1TP groups, with M1TN patients having significantly more total mesenchymal CTCs than M1TP patients (488.0 times more on average, p<0.0001, 90% CI 31.7 - 7510.5).

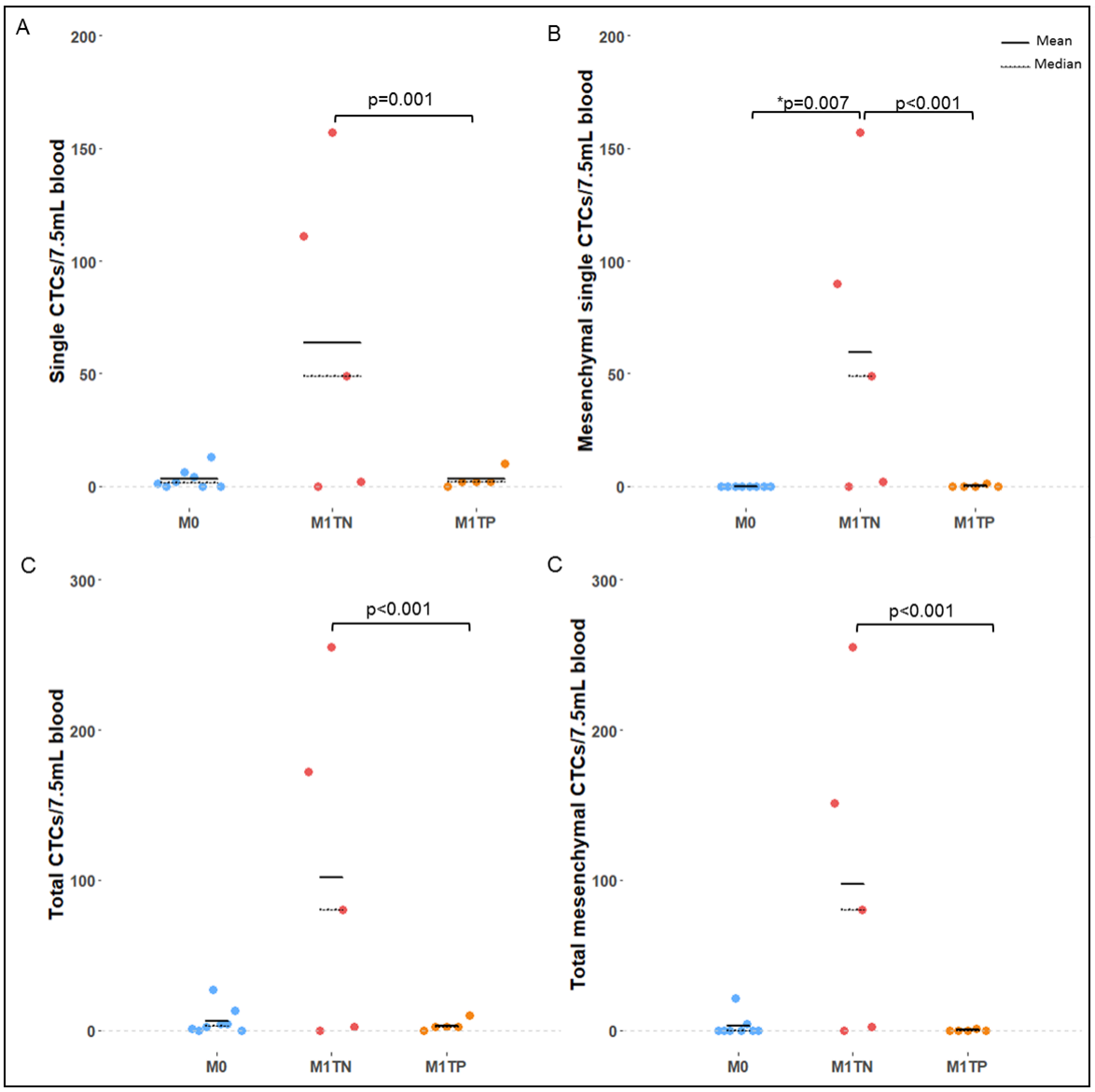

Figure 4. CTC counts comparisons between M0 (blue dots), M1TN (red dots), and M1TP (orange dots) patient groups.

A. Single CTC number per 7.5mL whole blood. **B.** Mesenchymal single CTCs number per 7.5mL whole blood. **C.** Total number of CTCs per 7.5mL whole blood. **D.** Total mesenchymal CTCs (single and CTCs in clusters) number per 7.5mL whole blood. Means

are represented as continuous black lines and medians are represented as dashed black lines. p-values obtained via negative binomial regression. *p-value corresponds to Fisher's test, where the presence/absence of CTCs was tested.

M1TN patients have significantly more single mesenchymal CTCs than both M0 (p=0.007) and M1TP patients (p<0.001). The M1TN group has an average of 59.6 (0-157) single mesenchymal CTCs vs. 0.2 (0-1) on the M1TP group, ie, M1TN patients have 297 times more single mesenchymal CTCs than M1TP patients on average (p<0.001, 90% CI 20.9-4231). Remarkably, there were no single mesenchymal CTCs detected in the M0 group but mesenchymal CTCs in clusters were found.

The average of single CTC counts was 3.3 (0-13), 63.8 (0-157), and 3.2 (0-10), respectively for M0, M1TN, and M1TP groups. M1TN patients have significantly more single CTCs than M1TP patients (63.8 times more on average, p=0.0012, 90% CI 19.8 - 205.3). Although more clusters were detected in the M1TN group than in the M0 group, the difference was not statistically significant.

Patients under antiplatelet therapy had significantly more single CTCs (p=0.025), total CTCs (p=0.029), and mesenchymal clusters (p=0.031) compared to patients who did not receive that therapy (Figure 5).

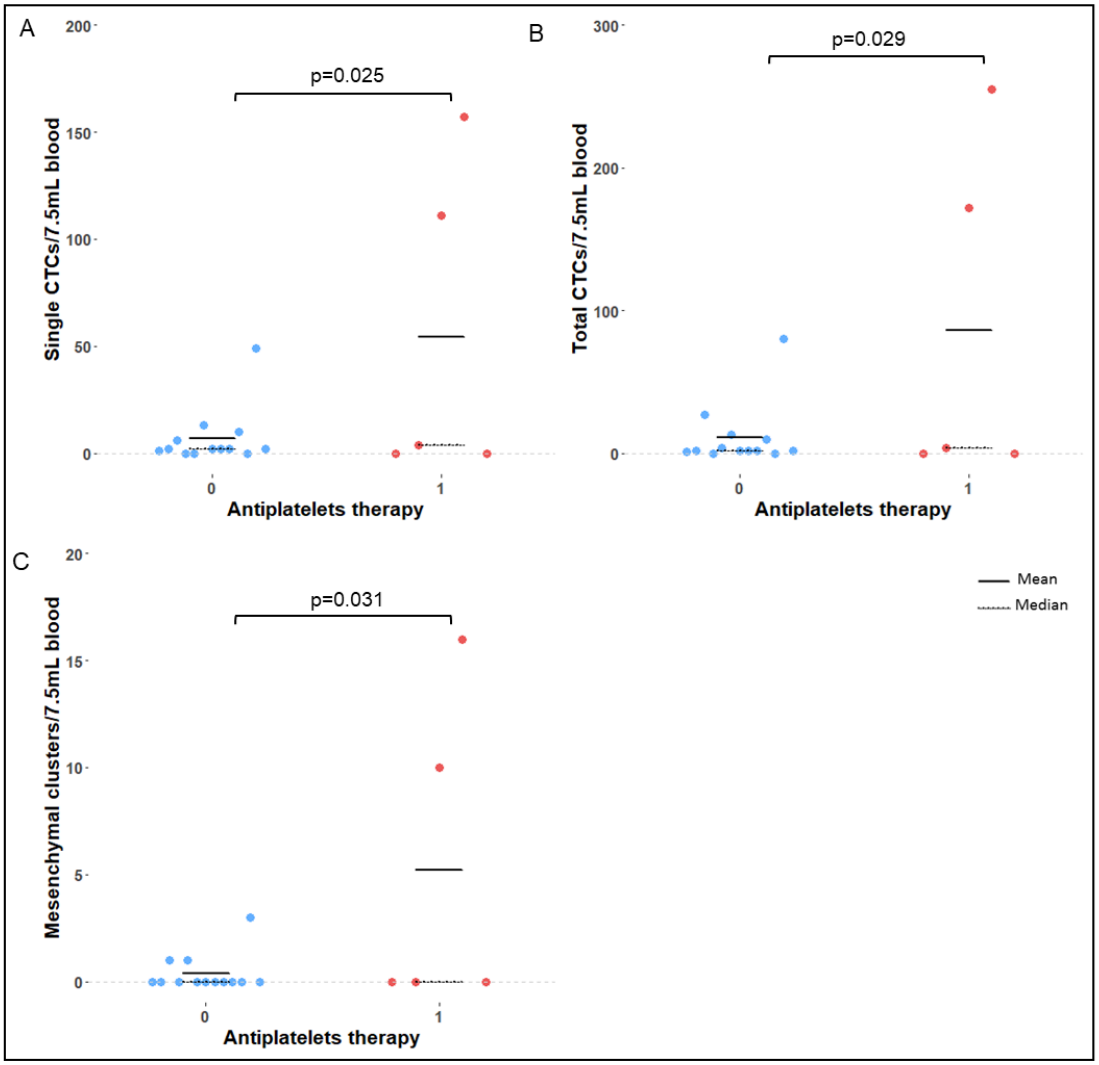

Figure 5. CTCs enumeration of single CTCs (A), total CTCs (B), and mesenchymal clusters (C) in patients under antiplatelet therapy (1) or not (0).

P-values obtained via negative binomial regression. The average is represented as a continuous black line and the median is represented as a dashed black line.

4.4.4. Correlation of clinical variables with CTC enumeration and phenotype

Despite the small number of samples analyzed, a strong positive correlation was found between CTC counts and INR, in both M0 and M1 groups. In the M0 group, INR correlates with mesenchymal CTCs in clusters and the total number of mesenchymal CTCs (r =0.85, p=0.008; and r = 0.85, p=0.008, respectively). In the M1 group, INR correlates with mesenchymal CTCs (in clusters r =0.970, p=0.001, single r =0.969, p=0.002, and total r=0.970, p=0.002), with CTC clusters (r=0.975,

p=0.001), with single CTCs (*r*=0.974, p=0.001), and with total number of CTCs (*r*=0.973, p=0.001).

Interestingly, in the M0 group, a negative correlation was found between epithelial CTCs and leucocyte count (*r*= -0.748, p=0.033), while in the M1 group, the correlation was positive (*r*= 0.80, p=0.005).

In the M0 group, a strong correlation was found between weight and mesenchymal CTCs (*r*= 0.828, p=0.011, and *r*= 0.828, p=0.011, respectively for mesenchymal CTCs in clusters and total mesenchymal CTCs), and between BMI and mesenchymal CTCs (*r*= 0.878, p=0.004, and *r*= 0.878, p=0.004, respectively in mesenchymal CTCs in clusters and total mesenchymal CTCs).

In the M1 group, increased leukocyte and neutrophil counts also correlated strongly with epithelial CTCs (*r*=0.801, p=0.005, and *r*=0.852, p=0.002, respectively). A moderate positive correlation was found in the entire cohort and in the M1 group between neutrophil-to-lymphocyte ratio (NLR) and total CTCs, single CTCs, mesenchymal CTCs, and CTC clusters.

Serum platelet counts also moderately and inversely correlated with total CTCs (*r*= -0.714, p=0.0203), single CTCs (*r*= -0.713, p=0.0206), CTC clusters (*r*= -0.715, p=0.0201), and mesenchymal CTCs in clusters (*r*= -0.714, p=0.0203), but only in M1 patients.

Serum albumin is moderately and inversely correlated to epithelial CTC counts in M1 patients (r= -0.83, p=0.01).

Serum hemoglobin levels are moderately correlated with total CTC counts in the M0 group (*r*= -0.747, p=0.033), but not in the M1 group.

Figure 6 shows the correlations found between CTCs and several clinicopathological variables.

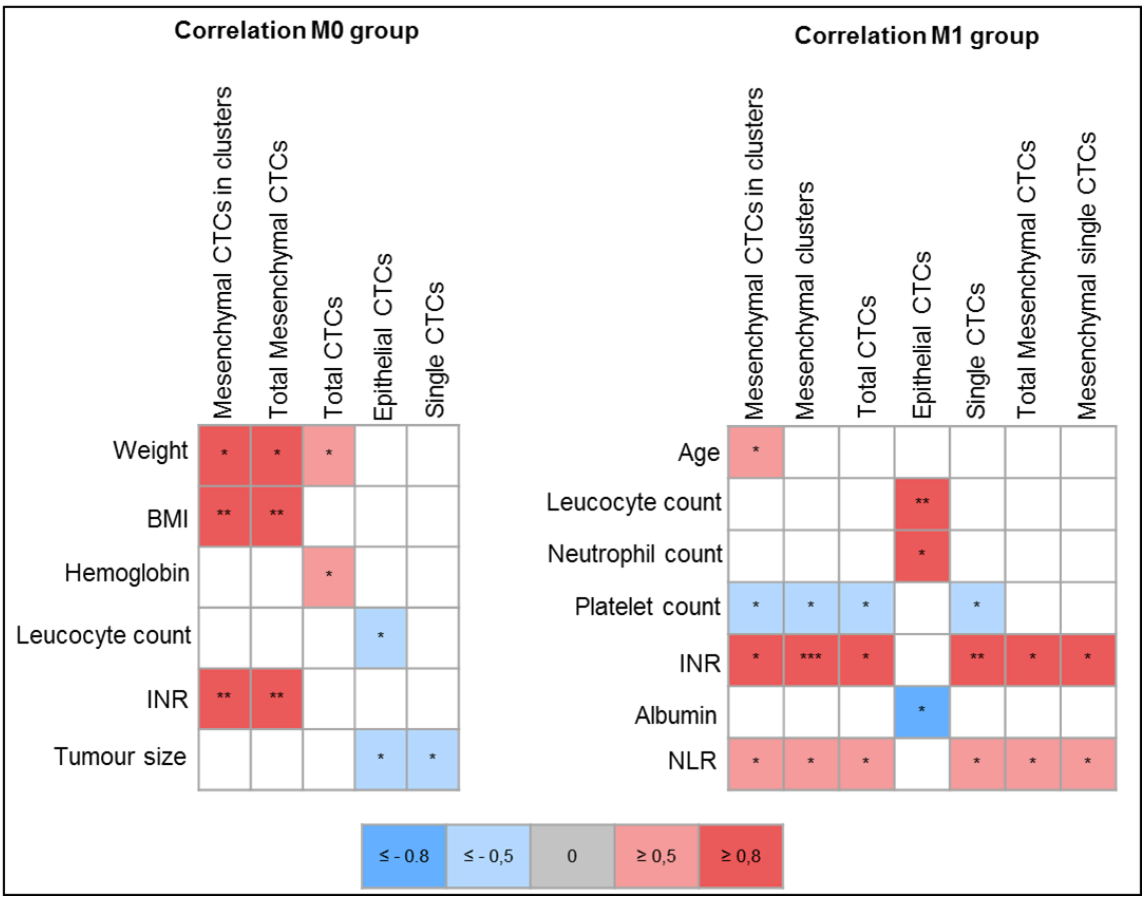

Figure 6. Correlations between CTC counts and clinical variables.

INR - international normalized ratio, NLR - neutrophil to lymphocyte ratio. Negative correlations are represented as light blue for moderate correlation ($r \geq -0.5$) and blue for strong correlation ($r \geq -0.8$). Positive correlations are represented as light red for moderate correlation ($r \geq 0.5$) and red for strong correlation ($r \geq -0.8$). p-values correspond to Pearson's correlation, * p<0.05, ** p<0.01, *** p<0.001.

4.4.5. Survival analysis

Overall survival (OS), in this study, was defined as the time from sample collection to death or last clinical follow-up of metastatic patients (M1TN and M1TP patients) (Figure 7). The median follow-up was 11.2 months. Patients with at least 5 total CTCs had a decreased overall survival compared to patients with less than 5 CTCs (HR 8.45, 95%CI 1.29 to 55.22, p=0.0143, figure 7A). This was also true for patients with at least 5 mesenchymal single and total CTCs (HR 7.657, 95%CI 0.717 to 81.78, p=0.0044, figure 7C-D), and for patients in whom CTC clusters

were identified (HR 0.1306, 95%CI 0.012 to 1.395, p=0.008). Clinical parameters like INR, BMI, and NLR did not affect OS.

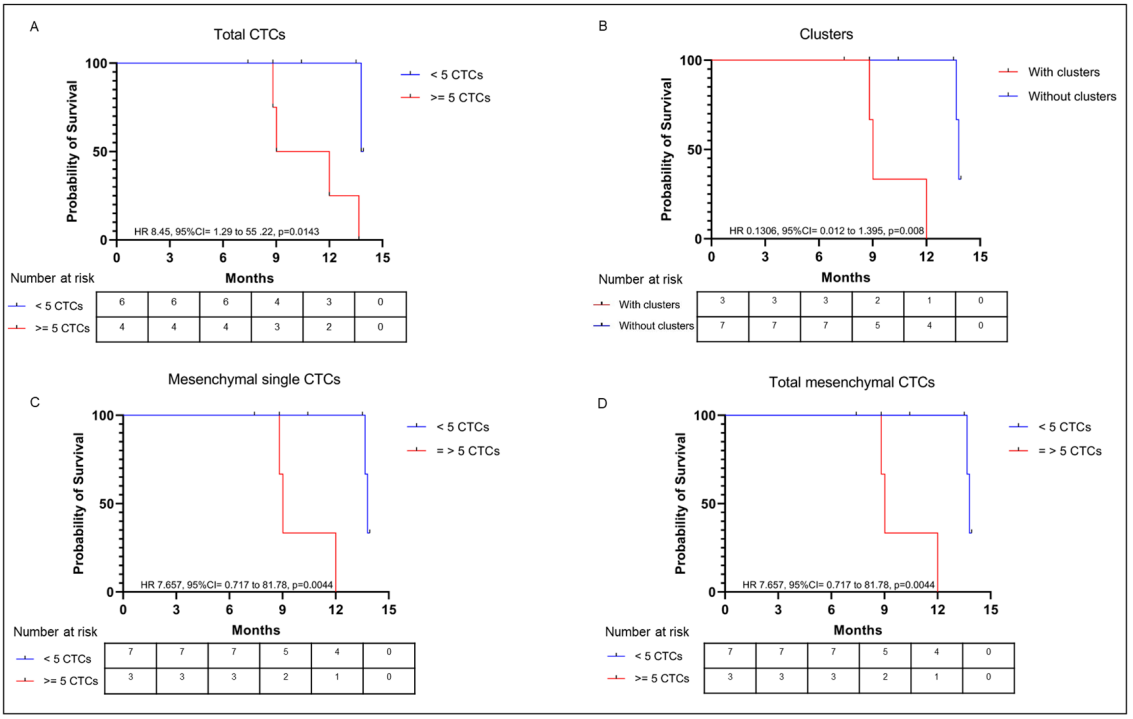

Figure 7. Kaplan-Meyer curves for overall survival (OS) of metastatic RCC patients for Total CTCs (A), Mesenchymal single CTCs (C), Total mesenchymal CTCs (D): less than 5 CTCs (blue line), and more or equal to 5 CTCs (red line); and for presence (red line) or absence (blue line) of CTC clusters (B).

4.5. DISCUSSION

4.5.1. Detection rates

The mean capture efficiency of RCC cells in the RUBYchip™ at the optimal flow rate of 80 µL/min was 74.9%. This high efficiency may be due to the absence of pre-processing of the blood samples, and to the design of the chip, with pre-filters to prevent micro-clots and obstruction, and microfilters designed to allow clearance of WBCs [36]. The geometry of the microfilters allows a good balance between the free passage of smaller and/or more deformable cells, typical of blood cells, and the entrapment of larger less deformable cells, like CTCs. CTCs are larger on average and have less deformability due to their larger nuclei, and high

cytoplasm-to-nucleus ratio [41]. However, there is wide variability in CTC sizes and phenotypes [42], which is why three RCC cell culture lines were tested in the present study. Consistent detection rates were found between the three cell culture lines at the determined optimal flow rate.

In a preclinical validation study of the RUBYchip™ conducted in metastatic breast cancer patients, CTC capture efficiency was up to 10 times higher compared to CellSearch® [36]. This was probably due to CellSearch®'s use of preservation tubes, to pre-processing of the blood sample, and the fact that it only targets CTCs expressing EpCAM. All these factors cause CTC loss.

The CTC detection rate in our RCC patient cohort was 77.8% overall, reaching 80% in M1 patients and 75% in M0 patients. This is a high detection rate compared to a median of 57% (IQR 55%) found in a systematic review of CTC detection techniques in RCC [4]. RCC is known to have lower CTC detection rates as compared to other cancer types, which is thought to be due to a greater prevalence of EMT and the consequent loss of the epithelial markers standardly used to identify these cells [4]. CellSearch® is the first technology approved by the FDA for the detection of CTCs and is seen as the benchmark in most epithelial cancers, except for RCC [43]. It is known that not all CTCs express EpCAM [44,45], and only 18.6% of RCC CTCs seem to express this marker [15].

The similarity of detection rates between localized and metastatic disease is an interesting finding. This shows that most cancer patients have CTCs, even in localized stages. It is the CTC counts that appear to vary with disease stage, not the proportion of patients with CTCs [4].

4.5.2. CTC counts

We found that M1TN patients had significantly higher total CTCs, single CTCs, mesenchymal CTCs, and total mesenchymal CTC counts compared to M1TP patients (figure 4A). M1TN patients have 31.8 times more total CTCs than M1TP patients (p=0.0003) and 15.9 more than M0 patients. These differences are substantial and mainly due to the increase in mesenchymal CTCs. M1TN patients

have 488 times more total mesenchymal CTCs than M1TP patients ($p<0.0001$) and 31 times more than M0 patients.

Compared to the M0 group, M1TN patients also appear to have significantly higher total CTC, single CTC, mesenchymal CTC, and total mesenchymal CTC counts, although statistical significance was only found for single mesenchymal CTCs ($p=0.007$). Epithelial CTCs were very similar between the groups, suggesting that disease stage did not affect its numbers. Interestingly we found no EMT CTCs in the entire cohort. This can be due to the low stage of the patients in the M0 group, whose cancer cells did not yet undergo EMT, and to the very advanced disease of M1 patients, where, hypothetically, all transitioning cancer cells had undergone full epithelial marker loss and concomitant gain of mesenchymal markers like vimentin.

M1TP patients did not have a significant difference in CTC counts compared to M0 patients. This may be a marker of some efficacy of systemic therapies in disease control and in limiting CTC release, despite the observed clinical progression.

These findings are in line with the literature. Another study also found higher CTC counts in patients with metastatic RCC compared to localized disease, of 9.6 vs. 5.3 CTC/7.5 mL, respectively [46]. Likewise, Liu S. et al. found that CTC counts were 2.2 times higher in late-stage (stage 3 and 4) compared to early-stage (stages 1 and 2) disease ($p<0.001$) [15]. The same authors also correlated mesenchymal CTCs with the RCC stage. Several other studies have demonstrated a correlation between CTC presence and staging, particularly with N+ and M+ status [10,15,22,29,46–49].

We found no difference in any of the CTC counts according to patient characteristics like N stage, smoking, obesity, hypertension, or diabetes. The study was underpowered for ECOG score, T stage, and tumor histological subtypes.

As previously stated, the majority of the CTCs in the M1TN groups are mesenchymal and only one M1 patient presented epithelial CTCs. This is compatible with the abundant EMT known to happen in advanced RCC. The

metastatic process is not yet completely understood, but it is generally accepted that EMT plays a role in CTCs release and is an important factor to explain tumor progression and treatment resistance [50]. A connection between EMT and disease aggressiveness, treatment response, and survival has been made in several cancer types [51,52].

We have considered DAPI+/CD45-/CK-/Vim+ cells to be mesenchymal CTCs. However, some controversy exists in the definition of mesenchymal CTCs. Vimentin is the most used marker for mesenchymal phenotyping, but other markers like N-cadherin, O-cadherin, Fibronectin, Serpin peptidase inhibitor, and Twist have been studied [53]. However, no marker or panel of markers has yet been found to definitively identify EMT or mesenchymal CTCs. Further characterization of these CTC subpopulations, for instance with downstream analysis using DNA and RNA sequencing, may improve our understanding of EMT and the role of these cells in cancer progression [54]. It has been suggested that some vimentin-positive cells could be circulating cancer-associated fibroblasts (cCAF) [55]. It has also been observed that metastatic CTCs are more viable when integrated into heterotypic clusters consisting of tumor and stromal cells [56]. We have found spindle-shaped vimentin-positive cells in some samples, which we considered as eventual cCAF and did not count as CTCs. We only considered as CTCs cells positive for vimentin and with specific cytomorphological features like round/ovoid shape, big nuclei, and high nucleus-to-cytoplasm ratio.

It is important to note that the first technology approved by the FDA for the capture and analysis of CTCs in a clinical setting in most cancers (CellSearch®) is heavily reliant on the expression of epithelial markers, which has been a hindrance to RCC CTCs research in the context of the widely present EMT in this particular cancer type. Hence, CTC isolation platforms capable of detecting EMT and mesenchymal CTCs, as well as CTC clusters, like the one used in this article, should be used in future RCC research to elucidate its biology and the clinical significance of these CTC subpopulations. In 2022, the Parsortix® microfluidic platform has also received FDA approval for metastatic breast cancer CTC detection, which confirms microfluidics has a promising technology.

4.5.3. CTC Clusters

CTC clusters can be defined as a group of more than 2 or 3 cancer cells and can have up to 100 cells [40,53]. Aceto *et al* found that CTC clusters have 23–50 times the metastatic potential of single CTCs, although they represent only 2% to 5% of all CTC events detected in a breast cancer mouse model [40]. Animal models have shown that CTC clusters arise from primary tumor vein invasion and fragmentation rather than an aggregation of single CTCs [57]. It has also been found that the injection of clustered cells resulted in reduced OS in mice compared to the injection of single CTCs (12.7 versus 15.7 weeks, $p < 0.016$) [40]. In the same mouse model, CTC clusters were cleared from circulation at least three times more rapidly than single CTCs (half-life: 6–10 min for clusters versus 25–30 min for single CTCs) [40].

In this cohort of patients, we found a higher average CTC cluster count in M1TN patients than in M0 and M1TP (figure 4B). Although the differences did not reach statistical significance, which was probably due to small sample size, they seem clinically relevant in this cohort. Particularly in the M1TP group we found no clusters, which may point to the efficacy of systemic treatment in preventing CTC clusters formation and release. Interestingly, all CTCs in these clusters were mesenchymal. CTC clusters seem to be more frequently comprised of mesenchymal CTCs rather than epithelial ones [54].

4.5.4. CTCs and survival outcomes

In this study, we found that patients with at least 5 total CTCs, mesenchymal CTCs (both single and total), and CTC clusters have a significantly lower OS. Several other studies have proven the impact of CTC presence and count on RCC survival [4,10,12,13,58–60]. Patients with CTC counts in the upper quartile, with > 0.12 CTCs/mL annually, had shorter overall survival (median 17.0 v 21.1 months, $p<0.001$) [58]. In another study, patients with mesenchymal CTCs had a slight decrease in survival (HR 1.2 (1.1-1.4, $p=0.005$) [61]. It was also found that postoperative total CTC counts higher than 6, post-operative mesenchymal CTC presence, and post-operative presence of CTC-white blood cell clusters significantly correlated with recurrence and metastasis [59]. In a study in M1 RCC, patients with total CTC counts of >3 had shorter OS, with a median of 13.8 versus

52.8 months on multivariate analysis (HR 1.67, 95%CI 0.95-2.93, p=0.003) [13]. There is still no standard cut-off for CTC counts to determine prognostic outcomes.

Similar findings were reported in other types of cancer. In colorectal cancer, patients with more than 3 CTCs/7.5 mL had reduced survival [62,63]. The risk of tumor progression and death was also higher in CTC-positive patients with pancreatic and esophageal cancer [64,65]. OS was also correlated to high CTC counts in a meta-analysis of gastric cancer [66].

4.5.5. CTC count correlations with clinical variables

A very strong positive correlation was found between all CTC counts and INR in metastatic patients, with the correlation being more moderate in patients with localized disease. This could be explained by the known prothrombotic state caused by CTCs and cancer in general, which may cause the consumption of coagulation factors and increased INR [67]. The EMT process can cause overexpression of tissue-factor in CTCs, conferring procoagulant properties which can contribute to metastasis formation [67,68]. However, in a study by Dirix, no significant association between activated partial thromboplastin clotting time (aPTT) or prothrombin time (PT) and CTC counts were encountered [69].

Patient weight and BMI showed strong positive correlations with mesenchymal CTC counts in the M0 group. Age showed a moderate positive correlation, consistent with all CTC counts. We can hypothesize that age may hinder immunity, which, associated with general patient frailty, can promote CTC survival.

CTCs in circulation interact with other blood cells and components. Hence, we looked for a relationship between CTC counts and other blood constituents.

We found that leukocyte counts are moderately positively correlated with epithelial CTCs in M1 patients, but inversely in M0 patients. This inversion was only observed for epithelial CTCs and not for mesenchymal CTCs or clusters. Tumor-associated neutrophils (TAN) appear to contribute to CTC survival by suppressing peripheral leukocyte activation in advanced cancer patients [70]. Additionally, single CTCs have been shown to have impaired interactions with T

lymphocytes and natural killer (NK) cells, therefore being protected against recognition by the immune system [71]. On the other hand, heterotypic CTC clusters have increased aggressiveness when conjugated with platelets, leukocytes, neutrophils, tumor-associated macrophages (TAM), and fibroblasts [71]. Also, metastasis-promoting gene expression profile changes were shown to occur with CTC and neutrophil interaction in a breast cancer mouse model study[71].

The neutrophil-to-lymphocyte ratio (NLR) correlated positively with total CTCs, single CTCs, mesenchymal CTCs, and CTC clusters in our cohort, particularly in the metastatic patients group. In our M1 group, increased neutrophil counts also correlated strongly with epithelial CTCs, but not with the other CTC variables. In a previous study by Peyton et al, elevation in absolute neutrophil count, and NLR >4 were independent predictors of decreased survival in RCC ($p<0.05$) [72].
In stage II/IV gastric cancer patients, CTCs detection was also significantly correlated with neutrophil count ($p=0.020$), and NLR ($p=0.009$) [73]. Therefore, NLR and neutrophil counts may prove to be surrogate predictors of survival in RCC.

In this study, CRP levels positively correlated with epithelial CTC counts. These findings appear to show that an elevation in inflammatory parameters correlates with increased CTC counts. In a paper on ovarian cancer, CRP was higher in CTC positive vs CTC negative patients, with a median of 4.33 (IQR 1.46-7.51) vs. 1.52 (IQR 0.50-4.50), $p=0.001$ [74]. In various types of cancer, CRP has been shown to have both prognostic value for predicting outcomes and the ability to predict response to chemotherapy [75]. A recent study has demonstrated a strong correlation in RCC between coagulation and both CRP and CTCs [76]. It is suggested that neutrophil extracellular traps may be the link between inflammation and coagulation.

In this cohort, the serum platelet count is inversely correlated to total CTC, single CTC, and CTC cluster counts, but only in metastatic patients. Some papers suggest that activated platelets can shield CTCs and protect them from immune destruction and blood flow shear forces [77–79]. CTC-coating platelets can

produce MHC-I-positive vesicles which may help CTCs escape recognition by NK and T cells [80]. This platelet recruitment and activation could lead to platelet consumption, decreasing their serum counts, which would help explain the inverse relation found in our cohort. A 2022 paper by Dirix et al also found a negative correlation between platelet count and CTC count in advanced breast cancer ($p < 0.0009$, R^2 0.167) [69]. On the other hand, Guan et al found a positive correlation between mesenchymal CTCs and platelet levels in RCC patients [61].

Platelet interaction with CTCs may also lead to EMT induction and maintenance through TGF-ß release, thereby promoting metastasis [81]. Platelet action, therefore, seems to promote CTC survival and metastasis. Further studies are needed to clarify the relationship between platelet and CTC counts.

We found serum hemoglobin levels to moderately and inversely correlate in the M0 group with total CTC counts. In a study of prostate cancer patients, a negative association between CTC counts and hemoglobin levels was found ($p=0.004$) [82]. Other studies have confirmed these associations [83,84].

Serum albumin also showed a moderate and inverse correlation in M0 patients, but only with epithelial CTC counts. In this cohort, patients with more advanced disease and higher tumor burden had poorer performance status, and concomitantly lower levels of albumin, as well as higher levels of CRP. Hypoalbuminemia is a surrogate marker of known disease processes present in advanced cancers, like increased catabolism, systemic inflammatory responses, increased vascular permeability and interstitial edema, and decreased albumin synthesis [85]. The correlation between serum albumin and CTCs is poorly studied. In a study on ovarian cancer, no difference in serum albumin levels was found between CTC+ and CTC- patients [74].

4.5.6. Study limitations and future directions

This study has limitations due to the small sample size, making it underpowered for most analyses. Despite the very limited clinical conclusions that can be drawn from such a small patient cohort, this study found a positive correlation between CTC counts and both staging and prognosis.

The future of liquid biopsies in cancer is promising and it is widely believed that liquid biopsies will play a crucial role in cancer diagnosis and in treatment decisions and monitoring in the coming years. Some of the benefits of liquid biopsies include their non-invasive nature, real-time monitoring capabilities, and ability to provide a comprehensive picture of the cancer cells and their behavior.

However, there are still some challenges that need to be addressed, such as the need for standardization and improved accuracy of liquid biopsy tests. Nevertheless, research and development in this field are ongoing and it is expected that liquid biopsy technology will continue to advance and become an increasingly important tool in the fight against cancer.

Further investigation is needed to identify effective molecular markers and develop reliable, standardized techniques for isolating and detecting CTCs in RCC so that they can be used as diagnostic, prognostic, and treatment management tools.

4.6. CONCLUSIONS

These findings show that the RUBYchip™ microfluidic size-based CTC detection device is an effective and reproducible way to isolate CTCs from RCC patients. It has high detection rates with short processing times due to fewer processing steps. The device identifies the different CTC phenotypes and also detects CTC clusters, aspects that are important in this type of cancer. This platform can be used in future RCC research to help improve our understanding of the metastatic process and disease progression, as well as to help guide patient management.

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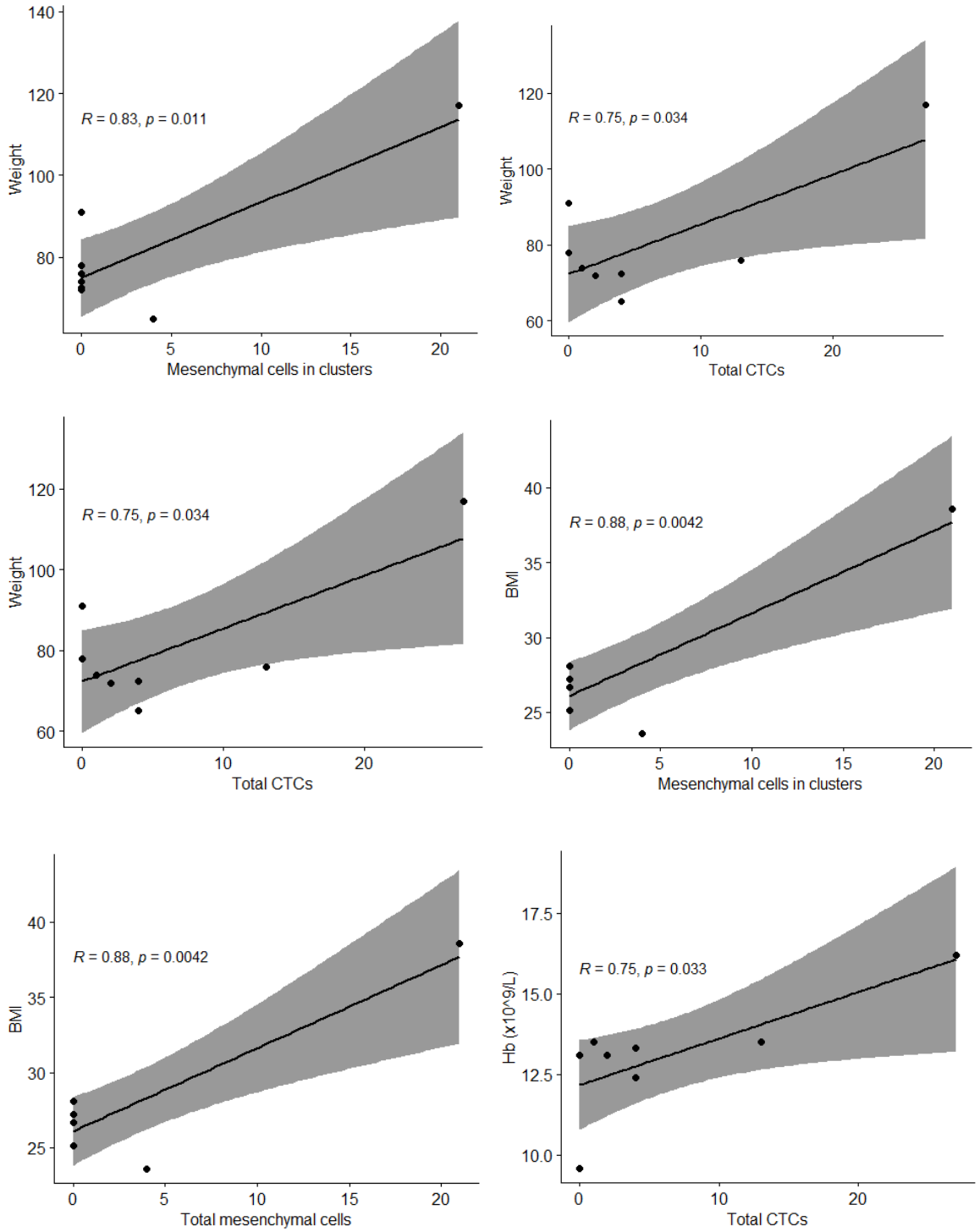
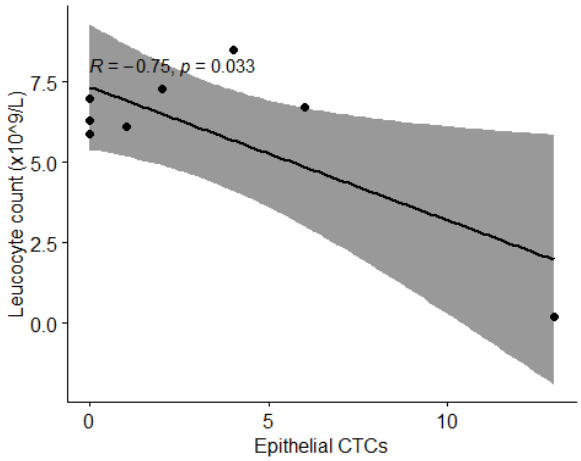
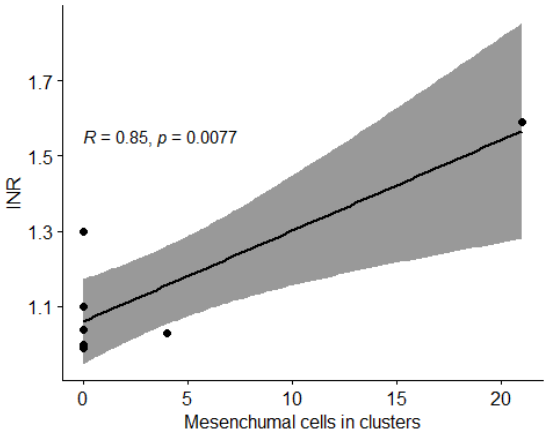
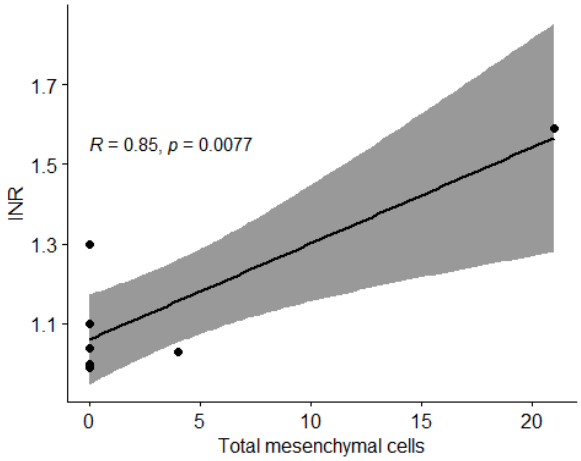
4.8. SUPPLEMENTS

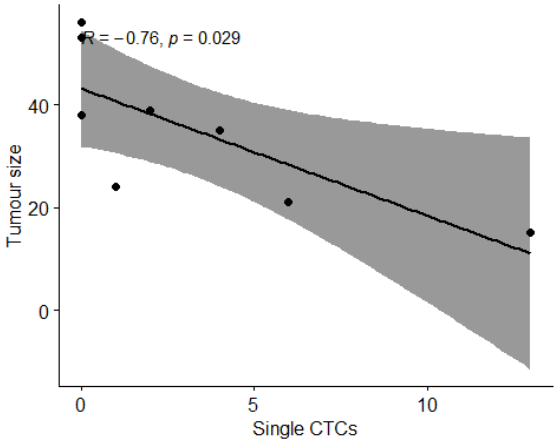
Figure 8. Correlation analysis plots for group M0




R = −0.75, p = 0.033
Leucocyte count (x10^9/L)
7.5
5.0
2.5
0.0
0
5
10
Epithelial CTCs


R = 0.85, p = 0.0077
INR
1.7
1.5
1.3
1.1
0
5
10
15
20
Mesenchumal cells in clusters


R = 0.85, p = 0.0077
INR
1.7
1.5
1.3
1.1
0
5
10
15
20
Total mesenchymal cells


R = −0.76, p = 0.029
Tumour size
40
20
0
0
5
10
Single CTCs

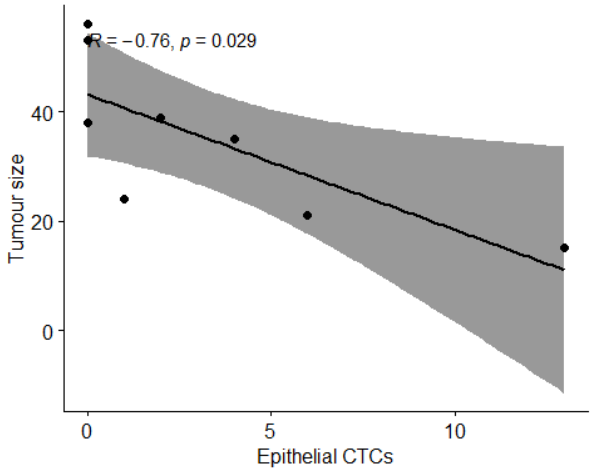
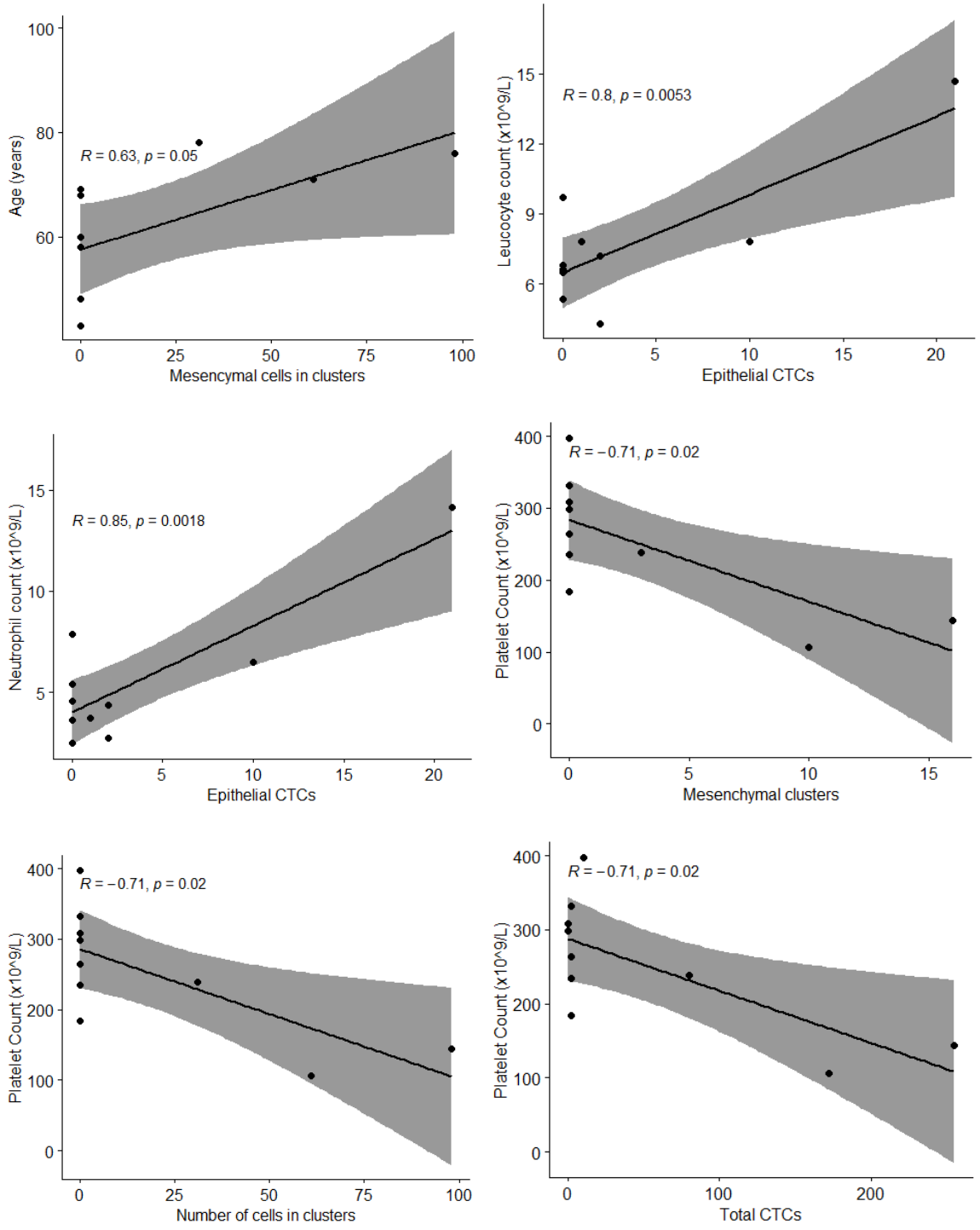
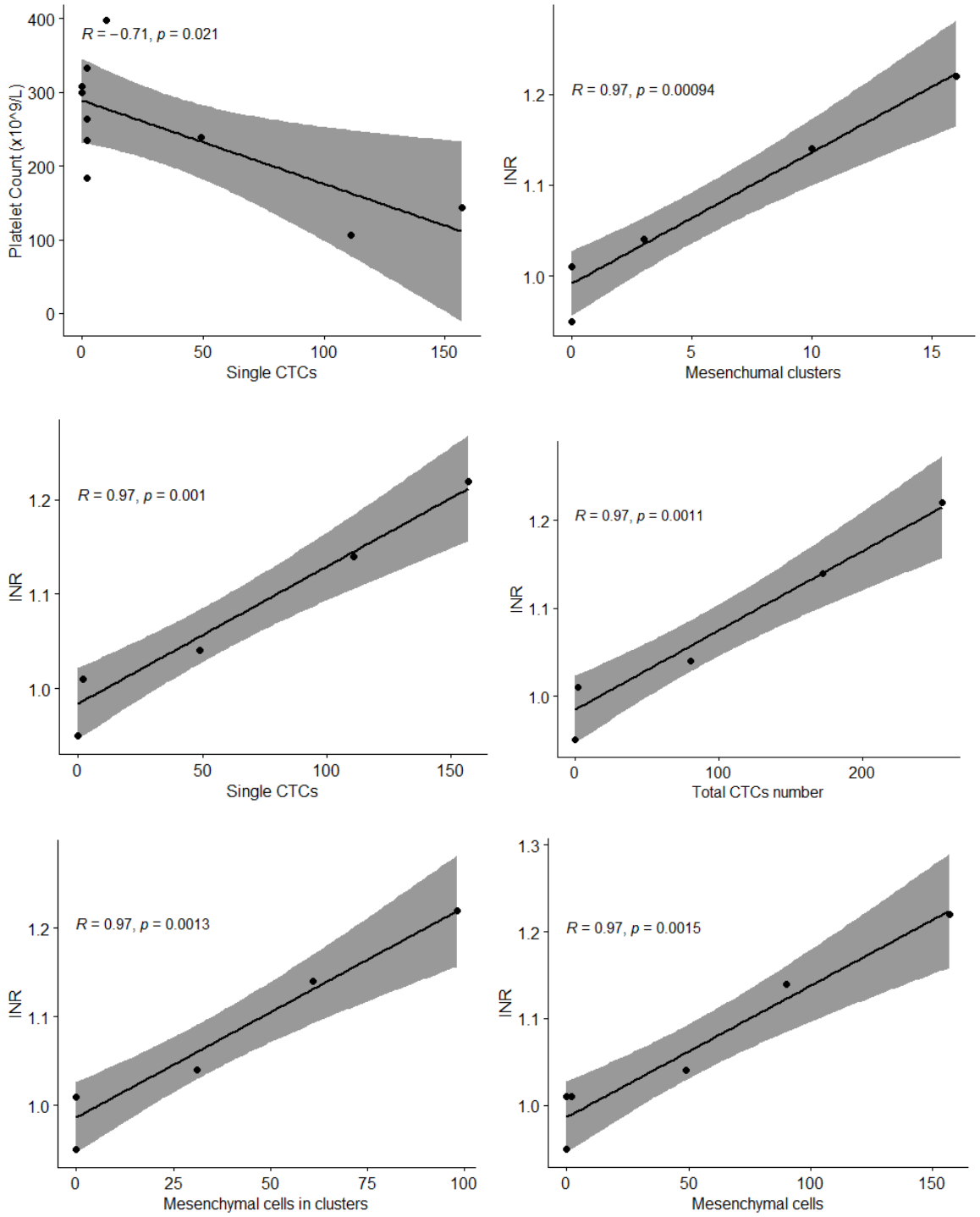
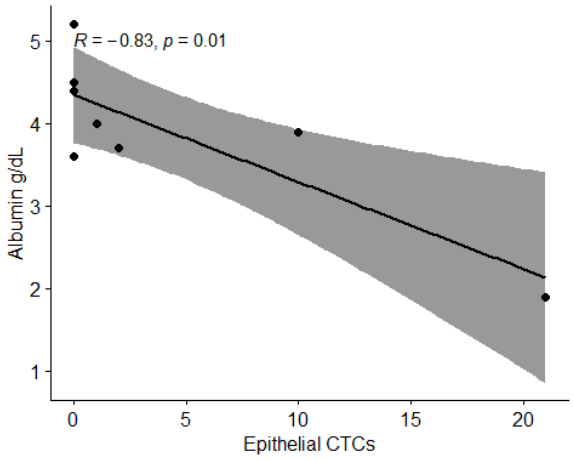
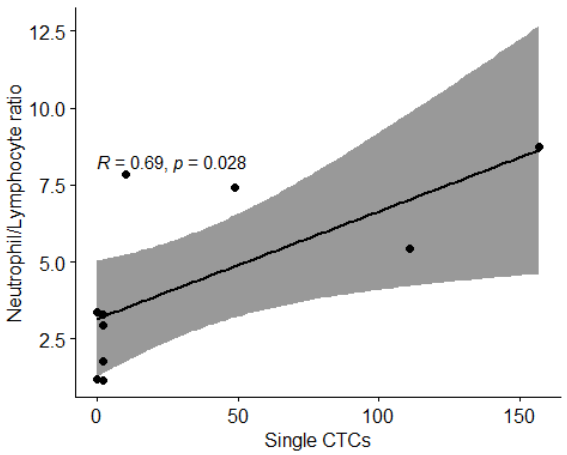

R = −0.76, p = 0.029
Tumour size
40
20
0
0
5
10
Epithelial CTCs

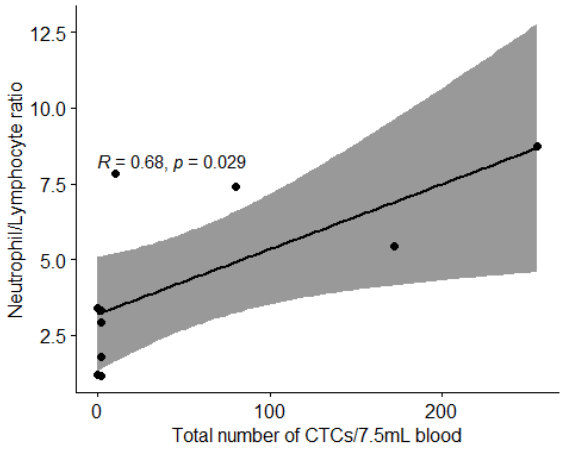
Figure 9. Correlation analysis plots for group M1

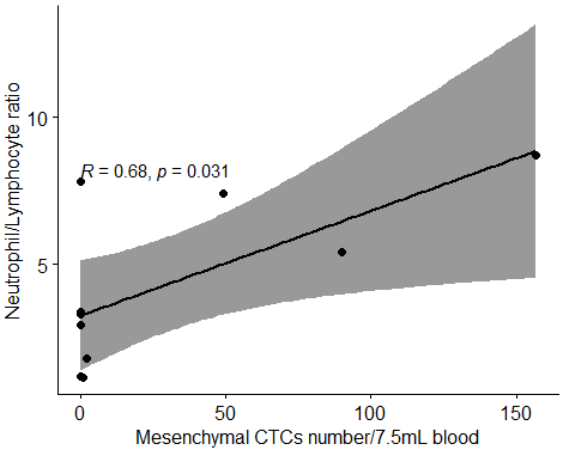


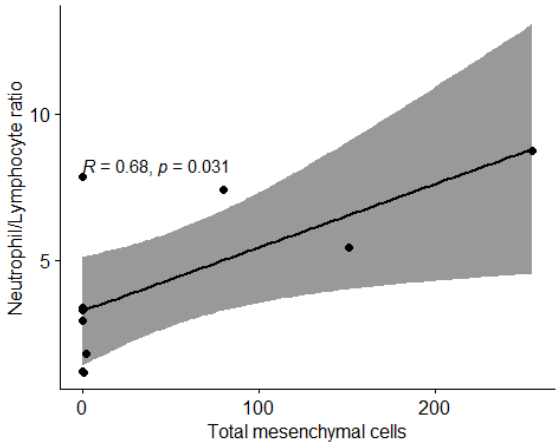

R = −0.71, p = 0.021
Platelet Count (x10^9/L)
Single CTCs
R = 0.97, p = 0.00094
INR
Mesenchumal clusters
R = 0.97, p = 0.001
INR
Single CTCs
R = 0.97, p = 0.0011
INR
Total CTCs number
R = 0.97, p = 0.0013
INR
Mesenchymal cells in clusters
R = 0.97, p = 0.0015
INR
Mesenchymal cells

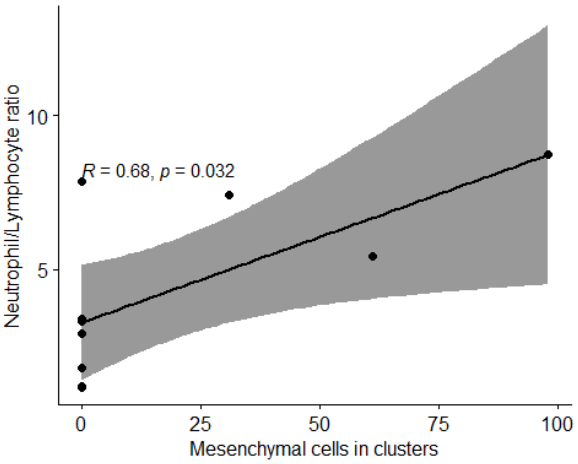

R = −0.83, p = 0.01
Albumin g/dL
Epithelial CTCs


R = 0.69, p = 0.028
Neutrophil/Lymphocyte ratio
Single CTCs


R = 0.68, p = 0.029
Neutrophil/Lymphocyte ratio
Total number of CTCs/7.5mL blood


R = 0.68, p = 0.031
Neutrophil/Lymphocyte ratio
Mesenchymal CTCs number/7.5mL blood


R = 0.68, p = 0.031
Neutrophil/Lymphocyte ratio
Total mesenchymal cells


R = 0.68, p = 0.032
Neutrophil/Lymphocyte ratio
Mesenchymal cells in clusters

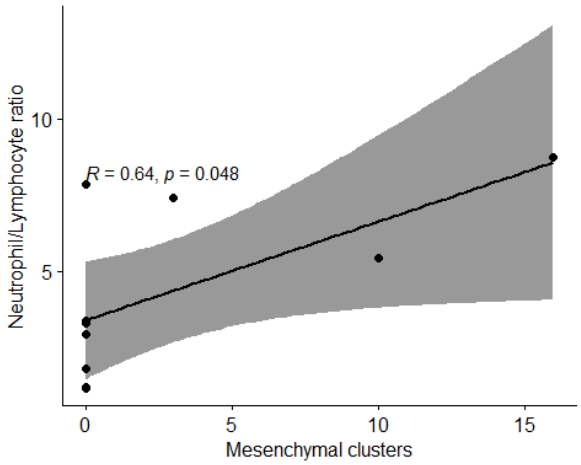

Neutrophil/Lymphocyte ratio
10
5
R = 0.64, p = 0.048
0
5
10
15
Mesenchymal clusters

Table 3. CTC counts and characterization per patient

Patient ID		#	Phenotyping		
			E	EMT	M
RCC001	Single CTCs	1	1	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	1	1	0	0
RCC002	Single CTCs	0	0	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	0	0	0	0
RCC003	Single CTCs	2	2	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	2	2	0	0
RCC004	Single CTCs	6	6	0	0
	Total CTC Clusters	1	0	0	21
	Total CTCs	27	6	0	21
RCC005	Single CTCs	4	4	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	4	4	0	0
RCC007	Single CTCs	0	0	0	0
	Total CTC Clusters	1	0	0	4
	Total CTCs	4	0	0	4

Patient ID		#	Phenotyping		
			E	EMT	M
RCC008	Single CTCs	13	13	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	13	0	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC018	Single CTCs	0	0	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	0	0	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC013	Single CTCs	111	21	0	90
	Total CTC Clusters	10	0	0	61
	Total CTCs	172	21	0	151

Patient ID		#	Phenotyping		
			E	EMT	M
RCC014	Single CTCs	0	0	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	0	0	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC015	Single CTCs	157	0	0	157
	Total CTC Clusters	16	0	0	98
	Total CTCs	255	0	0	255

Patient ID		#	Phenotyping		
			E	EMT	M
RCC016	Single CTCs	49	0	0	49
	Total CTC Clusters	3	0	0	31
	Total CTCs	80	0	0	80

Patient		#	Phenotyping

ID			E	EMT	M
RCC017	Single CTCs	2	0	0	2
	Total CTC Clusters	0	0	0	0
	Total CTCs	2	0	0	2

Patient ID		#	Phenotyping		
			E	EMT	M
RCC006	Single CTCs	0	0	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	0	0	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC009	Single CTCs	2	2	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	2	2	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC010	Single CTCs	2	2	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	2	2	0	0

Patient ID		#	Phenotyping		
			E	EMT	M
RCC011	Single CTCs	2	1	0	1
	Total CTC Clusters	0	0	0	0
	Total CTCs	2	1	0	1

Patient ID		#	Phenotyping		
			E	EMT	M
RCC012	Single CTCs	10	10	0	0
	Total CTC Clusters	0	0	0	0
	Total CTCs	10	10	0	0

Yellow, M0; green, M1 naive; blue, M1 progression

5. RESEARCH ARTICLE: A randomized controlled trial assessing the release of circulating tumor and mesenchymal cells in no-touch radical nephrectomy

5. RESEARCH ARTICLE: A randomized controlled trial assessing the release of circulating tumor and mesenchymal cells in no-touch radical nephrectomy

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5.1. ABSTRACT

Background: Circulating tumor cells (CTCs) may be the missing renal cell carcinoma (RCC) biomarker. All Robson principles of radical nephrectomy (RN) were rebutted, except for early pedicle ligation. No-touch (NT) resection showed benefit in other cancers.

Objective: To determine the impact of NT RN on CTC or circulating mesenchymal cell (CMC) release.

Design, setting, and participants:

Randomized controlled trial comparing NT vs. conventional (C) laparoscopic RN. Blood samples were collected at operation room arrival (S0), specimen extraction (S1), postoperative day 1 (D1), and 30 (D30). CTCs isolated and analyzed using the RUBYchip™.

Intervention(s): NT versus C laparoscopic RN.

Outcome measurements and statistical analysis: The primary endpoint was CTC and CMC count difference between groups and time points. Progression-free survival and complication rates were secondary endpoints.

Results and limitations: 34 patients were randomized from September 2021 to April 2022. No significant differences were found in CTC and CMC counts, variations between time points, complications, and survival outcomes between groups. CTC detection rates (DR) in NT and C groups were 0%, and 6.7% at both S0 and S1, 27.3% and 7.1% at D1, and 9.1% and 14.3% for D30, respectively. CMC DRs in each group were 58.3% and 73.3% at S0, 41.7% and 80.0% at S1, 36.4% and 57.1% at D1, and 45.5% and 42.9% at D30, respectively. A progressive decrease in CMCs was observed after surgery in the C group, mainly at D1 (4.78 to 1.64 CMCs/7.5mL blood, p=0.035). Healthy controls showed no circulating cells. High CMC counts were found in chronic inflammation controls and oncocytoma patients, not significantly different from RCC patients (p=0.460), questioning their CTC status. Limitations were the small sample size and no downstream cell analysis.

Conclusions: NT RN did not reduce circulating cell release nor improve survival compared to the C RN.

KEYWORDS: circulating mesenchymal cells, circulating tumor cells, kidney cancer, laparoscopy, liquid biopsy, microfluidics, no-touch surgery, radical nephrectomy, renal cell carcinoma.

5.2. INTRODUCTION

Renal cell carcinoma (RCC) had a global incidence of 431,288 in 2020[1]. Surgery is the preferred treatment for localized disease, although recurrence occurs in 20-40% of cases[2]. Radical nephrectomy (RN) is performed in half of the cases, whenever a partial nephrectomy is not possible[3]. Despite early detection significantly increasing in recent years, a third of RCC patients still present with metastatic disease[1,4].

Circulating tumor cells (CTCs) have been described as a potential RCC biomarker[5]. There is no clinically validated RCC biomarker currently available. CTCs have been shown to correlate with staging and survival in RCC[6–8]. Epithelial cell-adhesion molecule (EpCAM)-based CellSearch® was the first FDA-approved platform for CTC analysis, providing prognostic information in metastatic breast, prostate, and colorectal cancer[9]. However, the fact that only 18.6% of RCCs express EpCAM raised the need for alternative approaches for CTC analysis in RCC[6]. Maertens and colleagues reported that a cell-size-based system was the most efficient CTC isolation platform in RCC cell lines[10].

Exploratory studies of perioperative CTC kinetics in RCC revealed an increase in counts at D1 and a decrease on subsequent days[11–14]. Bluemke *et al.* found that pre-operative CTC detection had a relative risk of death of 2.7 (p=0.049), and postoperative detection of 4.3 (p=0.036)[13]. These results suggested that intraoperative tumor manipulation increases CTC release in RCC surgery and impacts prognosis.

The concept of no-touch (NT) tumor resection was first introduced in 1977 in colorectal cancer[15]. The NT group had less RT-PCR tumor mutations detection compared to those patients operated on using a conventional (C) technique (73% vs. 14%, respectively, p=0.05)[16]. However, two RCTs failed to demonstrate a

difference in outcomes for NT resection in colon cancer[17,18]. In a prospective lung cancer trial, CTC detection after surgery was significantly lower in an NT compared to a C approach (12.5 vs. 85.7%, p=0.02, respectively)[19].

RN was classically performed according to the five 1963 Robson principles[20]. All have been rebutted except for early renal pedicle ligation. A few publications described this technique as safe, although with no benefit in outcomes[21–23]. To our knowledge, there are no publications addressing the NT technique for RN.

The objective of this study was to investigate whether surgical manipulation of the kidney during RN increases circulating cell (CC) release and whether it can be reduced during RN by using an NT technique. The association between CC release and patient prognosis was a secondary objective.

5.3. MATERIALS (PATIENTS) AND METHODS

5.3.1. Study design and participants

A prospective randomized controlled trial was conducted at the Department of Urology of Centro Hospitalar Universitário Lisboa Norte (CHLUN) between September 2021 and April 2022 (Supplementary Figure S1). Patients with a renal mass and indication for laparoscopic RN (LRN) were enrolled. Exclusion criteria were patients with a history of another cancer, <18 years old, pregnant women, and surgically unfit patients. All patients gave written informed consent.

An NT LRN (NT group) was compared to a C LRN (C group).

Patients were positioned in lateral decubitus. Two 12mm ports were inserted, on the para-rectal and midclavicular lines, and two 5mm ports were placed, one in the midaxillary line, and the fourth 2cm away from the anterior superior iliac spine. An optional 5mm port was placed for liver retraction. The colon was retracted along the avascular plane just anterior to the Gerota's fascia until the vena cava on the right side and the aorta on the left side were exposed. The second part of the duodenum was reflected medially on the right side.

Hereafter, the surgical protocol for the NT group was to incise the Gerota's fascia just above the renal vein and to immediately and selectively ligate the renal pedicle using Hem-o-Lok® clips. No kidney manipulation was done until this point. The surgery then proceeded in the usual way.

In the C group, the surgical protocol entailed the identification and superior retraction of the ureter together with the kidney's lower pole, while the dissection continued cephalad until the renal pedicle was reached. The latter was then selectively ligated as previously described while maintaining renal traction.

Patients presenting for a total nephrectomy due to hypo-functioning kidneys and no history of cancer were used as controls and divided into two subgroups: 1) patients with systemic inflammation (inflammatory controls), defined by severe/recurrent pyelonephritis/pyonephrosis, elevated serum inflammatory parameters, and chronic pyelonephritis on pathology; and 2) patients with atrophic kidneys and none of the above criteria (healthy controls).

Study approved by the local ethics committee and conforming to CONSORT and the Declaration of Helsinki. Clinicaltrials.gov: NCT05070637.

5.3.2. Randomization and masking

Patients were randomly assigned to either group using a computer-generated allocation sequence. The allocation was disclosed to the surgical team upon patient's arrival at the operating room (OR).

5.3.3. Blood sample collection, CTC isolation, and characterization

A 7.5 mL peripheral venous blood sample was collected in EDTA tubes upon arrival to the OR (S0), after specimen extraction (S1), and at postoperative day one (D1), and 30 (D30). A single blood sample was collected from study controls on the day of enrollment.

Whole blood samples were anonymized, coded, and injected into the RUBYchip^{TM} microfluidic device at 80 µL/min, as described elsewhere[24]. The characterization of CCs was performed by immunocytochemistry using AF647-conjugated anti-human

vimentin (Vim; Biolegend, 1:50), PE-conjugated anti-human CD45 (Invitrogen, Thermo Fisher Scientific, 1:50), FITC-conjugated anti-human cytokeratin (CK), and DAPI (1 µg/mL). Fluorescent images were captured with an Allegro Plus (BioView, Israel) microscope at 20x magnification.

CCs were identified and characterized by morphological criteria (brightfield cell membrane integrity, round nucleus, cell-like morphology) and phenotypical criteria (DAPI+/CD45-/CK+ for epithelial CTCs, and DAPI+/CD45-/CK+/Vim+ for epithelial-mesenchymal transition CTCs). Circulating mesenchymal cells (CMCs) were identified as DAPI+/CD45-/CK-/Vim+. Groups of at least two cells with the above features were considered cell clusters.

5.3.4. Outcomes and statistical analysis

A sample size of 34 patients was calculated to detect a 20% decrease in CTCn variation after surgery in the NT group, assuming a Poisson distribution. The Kruskal-Wallis test was used to assess group homogeneity, followed by pairwise comparisons (Dunn's test). To compare CC counts between groups and time points, the Wilcoxon signed-rank test was used. The Mann-Whitney test was applied to compare absolute cell count differences between groups. The Wilcoxon rank-sum test was used to compare relative cell count differences between groups. Spearman’s correlation analysis was done between cell counts and quantitative clinical and imaging variables. Kaplan-Meier was used for progression-free (PFS), and overall survival (OS) analyses. Bonferroni corrections were used for multiple hypotheses testing. A significance level of 0.05 was considered. Statistical analyses were performed with R Software v2022.07.1 (R Foundation for Statistical Computing, Vienna, Austria).

5.4. RESULTS

Thirty-four patients were randomly assigned to the NT (n=18) and C (n=16) groups (Figure 1).

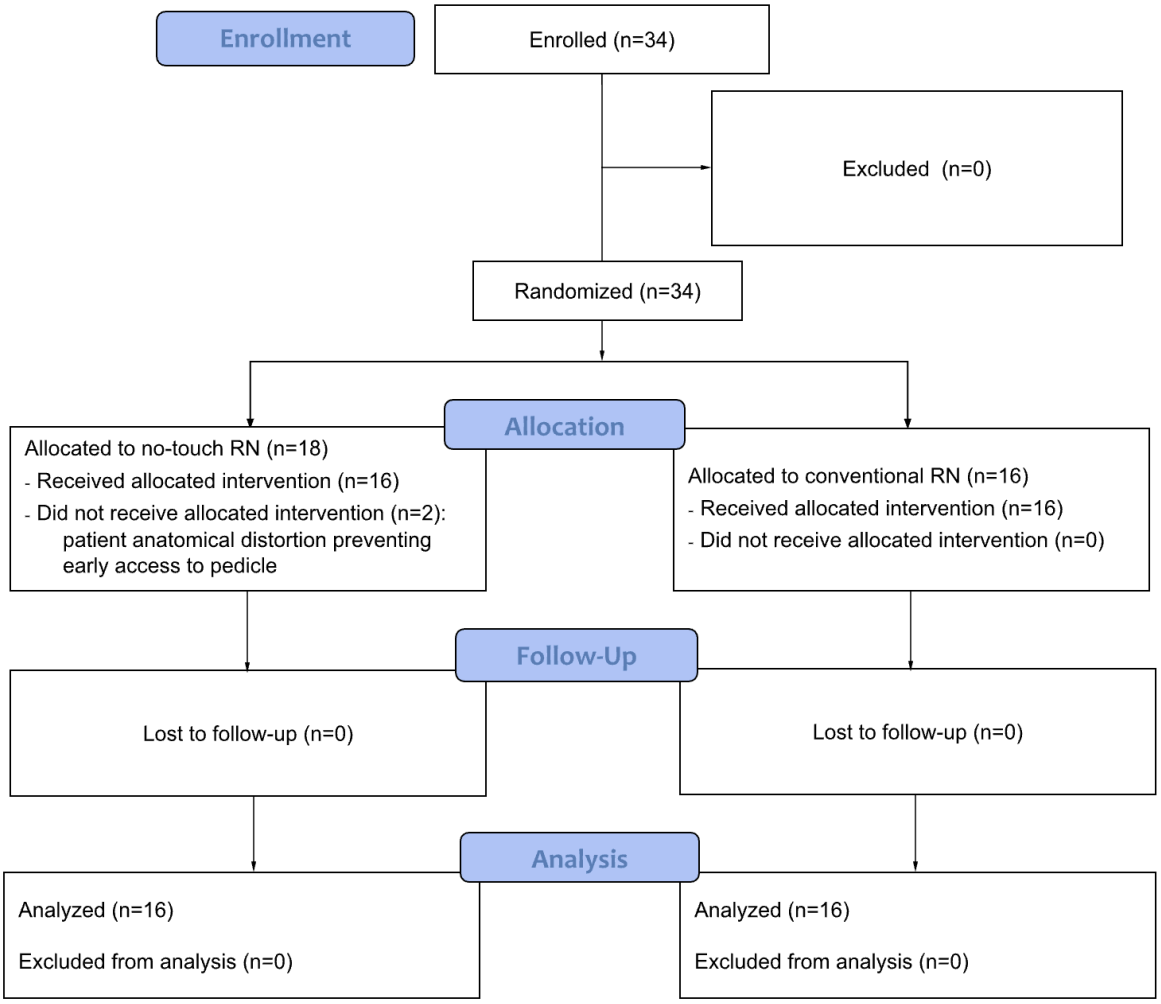

Figure 1. CONSORT flow diagram

Baseline clinicopathological characteristics of the study participants and controls are presented in Table 1.

Table 1. Clinicopathologic characteristics of the study population according to intervention groups and study controls

	NT group n=16	C group n=16	$p^{\dagger}$	Control group n=9	$p^{\pm}$

Age at surgery (yr), median (quartiles)	61.5 (56.8 - 70.5)	61.0 (55.8 - 71.0)	0.985	65 (55.0 - 69.0)	0.912
Gender M, n (%)	9 (56.25%)	13 (81.25%)	0.252	5 (80%)	0.693
BMI (kg/m2), median (quartiles)	26.2 (23.6 - 30.1)	26.9 (24.1 - 31.4)	0.509	29.3 (27.0 - 30.1)	0.637
Weight (kg), median (quartiles)	76.0 (68.8 - 88.5)	79.0 (69.5 - 98.5)	0.706	80.0 (77.5 - 91.0)	0.517
Height (cm), median (quartiles)	170.5 (161.2 - 177.0)	170.5 (166.8 - 176.8)	0.925	180.0 (170.0 - 181.0)	0.461
Smoking, n (%)	6 (37.5%)	4 (25%)	0.704	3 (33.3%)	1
Obesity, n (%)	5 (31.3%)	5 (31.3%)	1	2 (22.2%)	0.702
Hypertension, n (%)	11 (68.8%)	13 (81.3%)	0.685	6 (66.7%)	0.680
Diabetes, n (%)	2 (12.5%)	1 (6.25%)	1	4 (44.4%)	0.031
Antiplatelet Therapy, n (%)	5 (31.3%)	5 (31.3%)	1	2 (22.2%)	0.702
Anticoagulation, n (%)	1 (6.25%)	5 (31.3%)	0.172	0	0.309
ECOG, n (%)			0.654		0.028
0	6 (37.5%)	8 (50%)		2 (22.2%)	
1	7 (43.8%)	4 (25%)		5 (55.6%)	
2	3 (18.8%)	4 (25%)		0	
3	0	0		2 (22.2%)	
GFR (mL/min/1.73), median (quartiles)	64.5 (38.3 - 81.25)	81.5 (61.8 - 97.0)	0.239	73.0 (54.0 - 90.0)	0.935
Stage 1 (>90 mL/min), n (%)	3 (18.8%)	6 (37.5%)		3 (33.3%)	
Stage 2 (>60-89 mL/min), n (%)	6 (37.5%)	6 (37.5%)		2 (22.2%)	
Stage 3 (>30-59 mL/min), n (%)	4 (25%)	1 (6.25%)		3 (33.3%)	
Stage 4 (>15-29 mL/min), n (%)	1 (6.25%)	2 (12.5%)		1 (11.1%)	
Stage 5 (<15 mL/min), n (%)	2 (12.5%)	1 (6.25%)		0	
CKD History, n (%)			0.273		1
0	8 (50%)	11 (68.8%)		5 (55.6%)	
1	8 (50%)	4 (25%)		4 (44.4%)	
2	0	1 (6.3%)		0	
Dialysis, n (%)	3 (18.8%)	1 (6.3%)	0.600	0	0.559

Autoimmune disease, n (%)	3 (18.8%)	1 (6.3%)	0.600	0	0.559
Tumor characteristics:					
cT			1		1
cT0, n (%)	3 (18.8%)	2 (12.5%)		0	
cT1b, n (%)	5 (31.3%)	6 (37.5%)		0	
cT2a, n (%)	3 (18.8%)	4 (25%)		0	
cT2b, n (%)	2 (12.5%)	1 (6.3%)		0	
cT3a, n (%)	2 (12.5%)	3 (18.8%)		0	
cT3b, n (%)	1 (6.3%)	0		0	
cN			1		1
cN0, n (%)	16 (100%)	15 (93.75%)		0	
cN1, n (%)	0	1 (6.3%)		0	
cM			0.484		1
M0, n (%)	16 (100%)	14 (87.5)		0	
M1, n (%)	0	1 (6.3%)		0	
Mx, n (%)	0	1 (6.3%)		0	
Tumor side			0.716		0.039
Right, n (%)	11 (68.8)	9 (56.3%)		9 (100%)	
Left, n (%)	5 (31.3%)	7 (43.8%)		0	
Tumor size (mm), median (quartiles)	59.5 (40.8 - 98.3)	59.0 (49.0 - 75.5)	0.865	NA	NA
Peri operative characteristics:					
ASA, n (%)			0.767		1
I	1 (6.3%)	1 (6.3%)		0	
II	7 (43.8%)	6 (37.5%)		0	
III	8 (50%)	7 (43.8%)		0	
IV	0	2 (12.5%)		0	
Blood loss (ml), median (quartiles)	100.0 (50.5 - 212.5)	90.0 (50.0 - 375.0)	0.849	NA	NA
Peri operative blood transfusion, n (%)	0 (0)	0 (0)	0.317	NA	NA

Operative time (min), median (quartiles)	84.5 (63.8 - 95.3)	107.5 (96.3 - 131.8)	0.015 *	NA	NA
Time until renal vein exposure (min), median (quartiles)	21.0 (16.5 - 27.0)	37.0 (28.3 - 55.0)	0.001 *	NA	NA
Time between renal vein exposure and ligation (min), median (quartiles)	6.5 (3.0 - 13.5)	16.5 (13.0 - 32.3)	0.002 *	NA	NA
Days of hospital stay (days), median (quartiles)	2.5 (2.0 - 3.3)	3.0 (2.0 - 4.3)	0.405	NA	NA
Complications, n (%)	3 (18.8%)	3 (18.8%)	1	0	1
Clavien-Dindo classification, n (%)			1		1
I	0	1 (6.3%)		0	
II	2 (12.5%)	1 (6.3%)		0	
IVa	1 (6.3%)	1 (6.3%)		0	
Pathological parameters:					
Tumor maximum diameter (mm), median (quartiles)	50.0 (40.0 - 107.5)	64.5 (45.0 - 70.5)	0.890	NA	NA
Histologic type, n (%)			0.281		1
Clear cell	6	11		0	
Papillary type 1	0	1		0	
Papillary type 2	1	1		0	
Cromophobe	3	0		0	
Other RCC	2	2		0	
Oncocytoma	3	1		0	
Xantogranulomatous Pyelonephritis	1	0		0	
Pathology grade (Fuhrman), n (%)			0.793		1
1	3	2		0	
2	8	11		0	
3	0	0		0	
4	0	1		0	
Microvascular invasion, n (%)	2	3	1	0	1
Lymphatic invasion, n (%)	1	1	1	0	1

Renal vein (segmental) invasion, n (%)	0	4	0.106	0	1
Collecting system invasion, n (%)	1	1	1	0	1
Perirenal fat invasion, n (%)	3	3	1	0	1
pT, n (%)			0.208		1
pT1a	3	4		0	
pT1b	5	3		0	
pT2a	0	3		0	
pT2b	2	0		0	
pT3a	2	5		0	
pT3b	0	0		0	
pN, n (%)			NA		NA
pN0	4	6		0	
pN1	0	0		0	
Positive surgical margins, n (%)	0	0		NA	

* clinically significant; † comparison between no-touch and control groups; ± comparison between intervention and control groups. BMI, body mass index; C, control group; CKD, chronic kidney disease; ECOG, Eastern Cooperative Oncology Group performance status; GFR, glomerular filtration rate; NT, no-touch; M, male; min, minute; mL, milliliter; n, number; NA, not available; RCC, renal cell carcinoma.

Groups were balanced regarding all demographic and clinicopathological characteristics. The operative time was significantly shorter in the NT group compared to the C group (84.5 minutes [min], interquartile range [IQR] 31.5 vs. 107.5 min, IQR 35.5; p=0.015), as was the time to renal vein exposure (21.0 min, IQR 10.5 vs. 37.0 min, IQR 26.7 minutes; p<0.001).

Five patients had non-malignant histology: oncocytoma in four and focal xanthogranulomatous pyelonephritis (XP) in one. Only RCC patients were included for CTC and CMC cell counts in the intervention groups.

Baseline (S0) CC counts in intervention and control groups are shown in Table 2 and Figure 2.

Table 2. Baseline (S0) circulating cell counts and characterization in the intervention and control groups

	Control group n=9		Intervention group (S0) n=31									
	HC n=5	**IC** n=4	**RCC NT** n=12	**RCC C** n=15	**Oncocytoma** n=4	**IC vs. HC *p* value**	**IC vs. RCC C *p* value**	**IC vs. RCC NT *p* value**	**IC vs. O *p* value**	**O vs. HC *p* value**	**O vs. RCC C *p* value**	**O vs. RCC NT *p* value**
Single CTCs	0	0	0	0.33 (0,0,5)	0	1	1	1	1	1	1	1
Epithelial CTCs	0	0	0	0.33 (0,0,5)	0	1	1	1	1	1	1	1
EMT CTCs	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA
CMCs	0	22.5 (1,18.5,52)	3.0 (0,1.0,12)	8.47 (0,4.0,33)	24.75 (8,17.0,57)	0.017*	0.874	0.233	1	0.002*	0.205	0.037*
Single CMCs	0	11.75 (1,12.0,22)	2.58 (0,1.0,11)	7.2 (0,4.0,31)	14.25 (8,15.0,19)	0.018*	1	0.227	1	0.003*	0.371	0.052
CMCs in clusters	0	10.75 (0,6.5,30)	0.42 (0,0,3)	1.27 (0,0,10)	10.5 (0,2.0,38)	0.174	0.431	0.314	1	0.213	0.567	0.387
Clusters	0	2 (0,1.0,6)	0.17 (0,0,1)	0.4 (0,0,3)	2 (0,1.0,6)	0.202	0.520	0.360	1	0.202	0.520	0.360
Total CCs	0	22.5 (1,18.5,52)	3.0 (0,1.0,12)	8.8 (0,5.0,33)	24.75 (8,17.0,57)	0.017*	1	0.234	1	0.002*	0.269	0.030*
Single CCs	0	11.75 (1,12.0,22)	2.58 (0,1.0,11)	7.53 (0,5.0,31)	14.25 (8,15.0,19)	0.019*	1	0.225	1	0.003*	0.476	0.042*

Values are presented as means (minimun, median, maximum); * clinically significant; p-values calculated using the Kruskal-Wallis test for each cell count variable to assess the homogeneity of the four groups, followed by pairwise comparisons (using Dunn's test) with Bonferroni correction. C, conventional; CC, circulating cell; CMC, circulating mesenchymal cell; CTC, circulating tumor cell; EMT, epithelial-to-mesenchymal transition; HC, healthy control; IC, inflammatory control; n, number; NA - not available; NT, no-touch; RCC, renal cell carcinoma; O - oncocytoma; S0, OR arrival time-point.

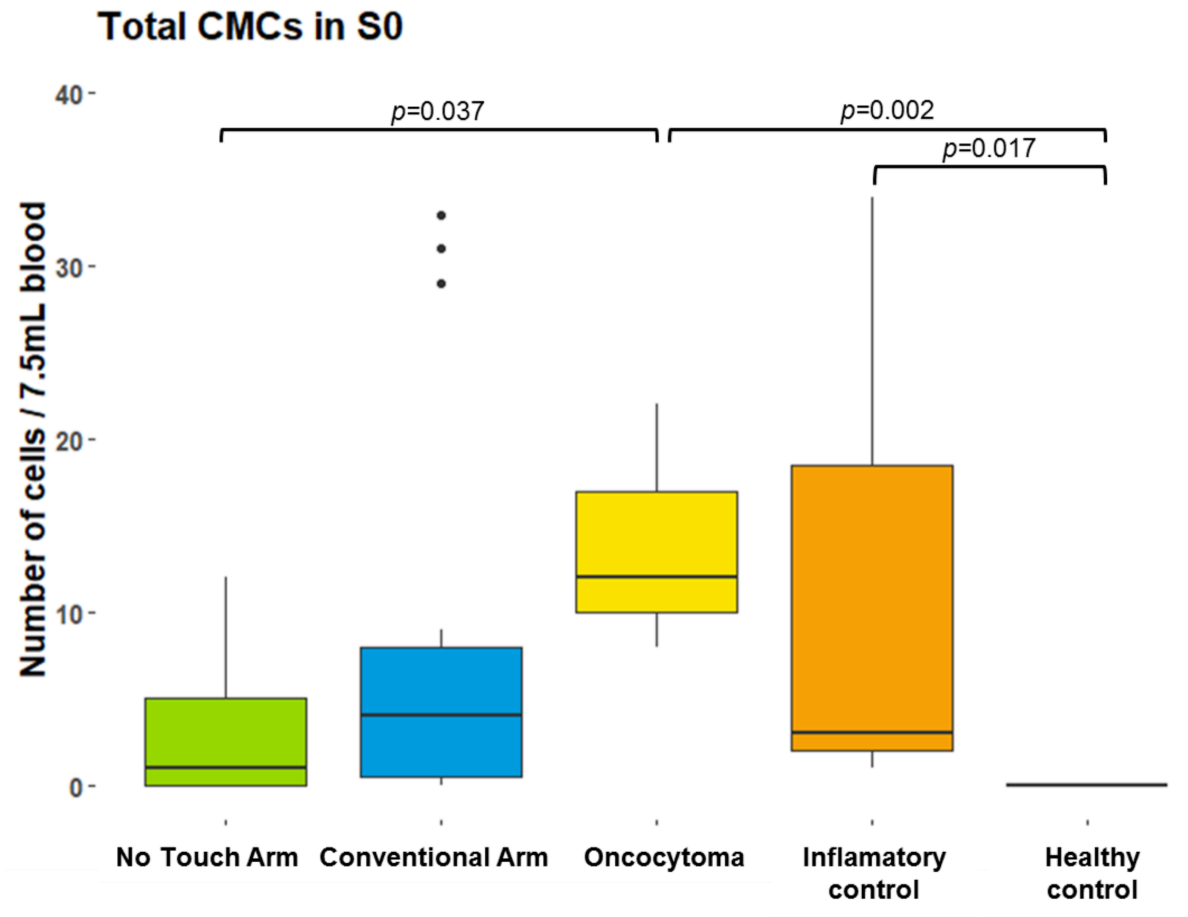

Figure 2. Box-plots comparing total CMC counts at S0 between NT group, C group, oncocytoma, inflammatory controls, and healthy controls.

CMC, circulating mesenchymal cells; NT, no-touch; S0, baseline (arrival at the operating room).

No CTCs were detected in the control groups and in oncocytoma patients. No CMCs were detected in the healthy control group (Figure 2). However, all patients in the chronic inflammation control group had a significant number of CMCs with a mean of 22.5 (range 1-52) cells per 7.5 mL of blood (p=0.007 compared to healthy controls), with no difference to cancer patients in the intervention groups (p=0.291 and 0.460, for NT and C groups, respectively). CMCs were also detected in all oncocytoma patients, with no difference to the RCC C group (p=1.000) or chronic inflammation patients (p=0.205), but with higher counts compared to the RCC NT group (p=0.037).

No correlation was found between CMC counts and serum inflammatory parameters, namely C-reactive protein, leucocyte, and neutrophil counts.

Table 3 shows the CC counts and characteristics in each intervention group at each considered time point.

Table 3. CC counts and characterization in each study group and time point.

	RCC NT group (n=12)				**RCC C group (**n=15)			
	S0	**S1**	**D1**	**D30**	**S0**	**S1**	**D1**	**D30**
Single CTCs	0	0	1.36 (0,0,13)	0.09 (0,0,1)	0.33 (0,0,5)	0.07 (0,0,1)	0.07 (0,0,1)	0.36 (0,0,4)
Epithelial CTCs	0	0	0.36 (0,0,2)	0.09 (0,0,1)	0.33 (0,0,5)	0.07 (0,0,1)	0.07 (0,0,1)	0.36 (0,0,4)
EMT CTCs	0	0	1.00 (0 0,11)	0	0	0	0	0
CMCs	3.00 (0,1.00,5)	2.00 (0,0,11)	1.91 (0,0,12)	0.91 (0, 0, 7)	8.47 (0,4.00,33)	7.00 (0,4.00,31)	1.43 (0,1.00,6)	1.57 (0,0.50,11)
Single CMCs	2.58 (0,1.00,11)	2.00 (0,0,11)	1.91 (0,0,12)	0.45 (0, 0, 2)	7.20 (0,4.00,31)	6.27 (0,4.00,22)	1.43 (0,1.00.6)	1.21 (0,0.50,6)
CMCs in clusters	0.42 (0,0,3)	0	0	0.45 (0, 0, 5)	1.27 (0,0,10)	0.73 (0,0,11)	0	0.36 (0,0,5)
Clusters	0.17 (0,0,1)	0	0	0.18 (0, 0, 2)	0.40 (0,0,5)	0.20 (0,0,3)	0	0.07 (0,0,1)
Total CCs	3.00 (0,1.00,12)	2.00 (0,0,11)	7.00 (0,1.00,25)	1.00 (0, 0, 8)	8.80 (0,5.00,33)	7.07 (0,4.00,31)	1.5 (0,1.00,6)	1.93 (0,1.00,11)
Single CCs	2.58 (0,1.00,11)	2.00 (0,0,11)	3.27 (0,1.00,25)	0.55 (0, 0, 2)	7.53 (0,5.00,31)	6.33 (0,4.00,22)	1.5 (0,1.00,6)	1.57 (0,1.00,6)

	Oncocytoma (n=4)				**Xanthogranulomatous Pyelonephritis (**n=1)			
	S0	**S1**	**D1**	**D30**	**S0**	**S1**	**D1**	**D30**
Single CTCs	0	0	0	0	0	0	0	0
Epithelial CTCs	0	0	0	0	0	0	0	0
EMT CTCs	0	0	0	0	0	0	0	0
CMCs	24.75 (8,17.00,57)	13.5 (1,11.00,31)	0.75 (0,0.50,2)	5.50 (0 6.00,12)	4	0	1	1
Single CMCs	14.25 (8,15.00,19)	6.25 (1,6.00,12)	0.75 (0,0.50,2)	4.75 (0,5.00,9)	4	0	1	1
CMCs in clusters	10.50 (0,2.00,38)	7.25 (0,1.00,27)	0	0.75 (0,0,3)	0	0	0	0
Clusters	2.00 (0,1.00,6)	0.50 (0,0.5,1)	0	0.25 (0,0,1)	0	0	0	0
Total CCs	24.75 (8,17.00,57)	13.50 (1,11.00,31)	0.75 (0,0.50,2)	5.50 (0,6.00,10)	4	0	1	1
Single CCs	14.25 (8,15.00,19)	6.25 (1,6.00,12)	0.75 (0,0.50,2)	4.75 (0,5.00,9)	4	0	1	1

Values presented as means (minimum, median, maximum); S0, OR arrival time-point; S1, specimen extraction time-point; D1, postoperative day 1 time-point; D30, postoperative day 30 time-point; C, conventional; CC, circulating cells; CMCs, circulating mesenchymal cells; CTCs, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; NT, no-touch; RCC, renal cell carcinoma.

The total CC detection rates in the NT, C, and overall RCC groups were 58.3%, 80.0%, and 70.4% at S0, 41.6%, 86.7%, and 66.7% at S1, 50.0%, 64.3%, and 60.0% at D1, and 54.5%, 42.9%, and 44.0% at D30, respectively.

The CTC detection rates in the NT, C, and whole RCC groups were 0%, 6.7%, and 3.7%, on both S0 and S1, 27.3%, 7.1%, and 16.0% on D1, and 9.1%, 14.3%, and 12.0% on D30, respectively.

Most CCs were CMCs in all groups and time points. Single CMCs were found in all time points and every group, in one patient with focal XP at S1. CMC clusters were found in every group at all time points except at D1, and in the NT and XP groups at S1.

The CMC detection rate in the RCC NT, RCC C, and whole RCC groups was 58.3%,73.3%, and 81.5% at S0, 41.7%, 80.0%, and 63.0% at S1, 36.4%, 57.1%, and 48.0% at D1, and 45.5%, 42.9%, and 44.0% D30, respectively.

Clusters of CMCs were found in 16.7%, 20.0%, and 18.5% of patients in the NT, C, and whole RCC groups at S0, 0%, 6.7%, and 3.7% at S1, 0%, 0%, and 0% at D1, and 13.3%, 0.0%, and 7.4% at D30.

A progressive decline in total CCs and CMCs after surgery was observed in both intervention groups, but it was only significant in the C group (Figure 3). This decrease was mainly due to the significant decline in CMCs at D1 (from 4.78 to 1.64 CMCs/7.5 mL of blood; p=0.035) since no differences were found between S0 and S1 nor between D1 and D30. In all intervention groups, CMC clusters disappeared at D1 and reappeared at D30.

Table 4 shows CC counts and variation between intervention groups and time points.

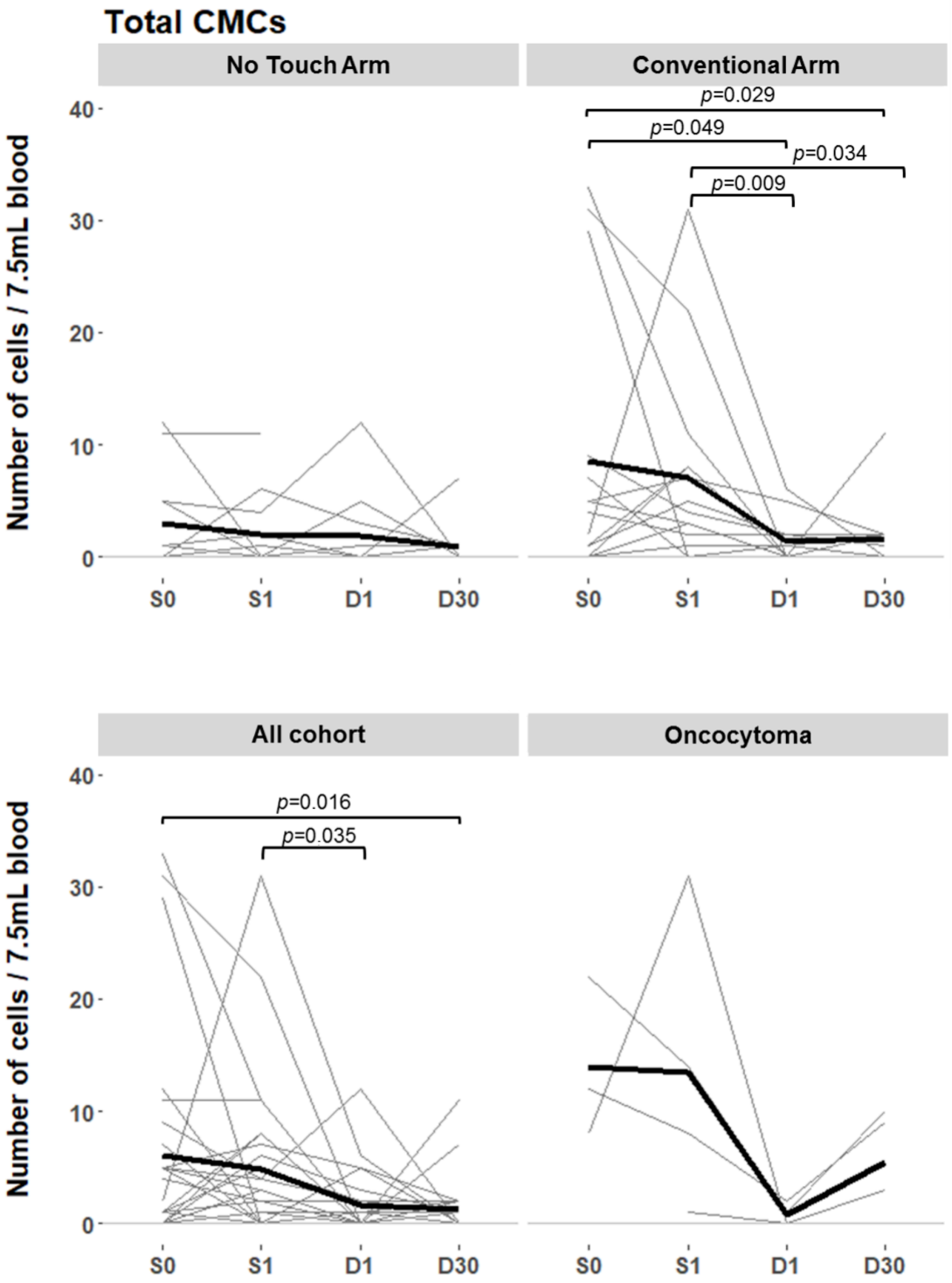

Figure 3. CMC counts at different time points (S0, S1, D1, and D30) for the NT and C groups (top figure), and for the entire cohort and oncocytoma groups (bottom figure). Individual patients are represented as grey lines and the mean is represented as a black line. S0 - blood sample collected at OR arrival; S1 - blood sample collected at specimen extraction; D1 - blood sample collected at postoperative day 1; D30 - blood sample collected at postoperative day 30; NT - no-touch group; C - conventional group; CMCs - circulating mesenchymal cells.

Table 4. CTC counts and variation in each intervention group and time point (primary outcome)

	S0			S1			D1			D30			Cell count difference S1 - S0			Relative cell count difference S1 - S0 (%)			Cell count difference D1 - S0			Relative cell count difference D1 - S0 (%)		
	NT	C	*p**	NT	C	*p**	NT	C	*p**	NT	C	*p**	NT	C	*p*±	NT	C	*p*±	NT	C	*p*±	NT	C	*p*±
Single CTCs	0	0.33	0.371	0	0.07	0.371	1.36	0.07	0.169	0.09	0.36	0.662	0	-0.26	0.412	NA	-80	NA	1.36	-0.26	0.126	NA	-78.6	NA
Epithelial CTCs	0	0.33	0.371	0	0.07	0.371	0.36	0.07	0.169	0.09	0.36	0.662	0	-0.26	0.412	NA	-80	NA	0.36	-0.26	0.126	NA	-78.6	NA
EMT CTCs	0	0	NA	0	0	NA	1	0	0.259	0	0	NA	0	0	NA	NA	NA	NA	1	0	0.295	NA	NA	NA
CMCs	3.00	8.47	0.251	2.00	7.00	**0.033**	1.91	1.43	0.554	0.91	1.57	0.302	-1.00	-1.47	0.883	-33.3	-17.3	0.268	-1.09	-7.04	0.754	-36.36	-83.1	0.311
Single CMCs	2.58	7.20	0.203	2.00	6.27	**0.033**	1.91	1.43	0.554	0.45	1.21	0.235	-0.58	-0.93	0.677	-22.6	-13.0	0.268	-0.67	-5.77	0.213	-100.0	-100.0	0.311
CMCs in clusters	0.42	1.27	0.746	0.00	0.73	0.371	0.00	0.00	NA	0.45	0.357	0.861	-0.42	-0.54	1	-100.0	-42.1	NA	-0.42	-1.27	0.784	-100.0	-100.0	NA
Clusters	0.17	0.40	0.719	0.00	0.20	0.371	0.00	0.00	NA	0.18	0.07	0.846	-0.17	-0.20	1	-100.0	-50.0	NA	-0.17	-0.40	0.213	-100.0	-100.0	NA
Total CCs	3.00	8.80	0.142	2.00	7.07	0.021	7.00	1.50	0.775	1.00	1.93	0.373	-1.00	-1.73	0.961	-33.3	-19.7	0.283	4.00	-7.30	0.077	9.1	-83.0	0.644
Single CCs	2.58	7.53	0.106	2.00	6.33	0.021	3.27	1.50	0.775	0.55	1.57	0.270	-0.58	-1.20	0.826	-22.6	-15.9	0.283	0.69	-6.03	0.077	26.7	-80.1	0.607

Values presented as means; * Kruskal-Wallis test; ± Wilcoxon test.

S0, blood sample collected at OR arrival; S1, blood sample collected at specimen extraction; D1, blood sample collected at postoperative day 1; D30, blood sample collected at postoperative day 30; NT, no-touch group; C, conventional group; CC, circulating cell; EMT, epithelial-to-mesenchymal transition; CMC, circulating mesenchymal cell; CTC, circulating tumor cell; NA, impossible to determine.

No significant differences were found between intervention groups in absolute and relative CTC and CMC variations between S0 and the remaining time points, suggesting that there is no reduction in CTC or CMC release with NT LRN. However, a significantly lower CMC count was found in the NT arm compared to the C arm at S1 (4.38 vs. 7.06, p=0.044). There were no differences in CC counts at any other time point.

In the whole RCC cohort, moderate negative correlations were found between CT tumor contrast washout and CMCs at S1 (r=-0.503, p=0.008, and r=-0.425, p=0.027, for corticomedullary and late phases respectively), and CMC clusters at S0 (r=-0.526, p=0.005, and r=-0.442, p=0.021, for corticomedullary and late phases, respectively). Thus, the greater the washout, the fewer CMCs (Supplementary Material).

Total CC counts in S0 and ∂S1-S0 correlated with MAP score[27] (p=0.031 and 0.020, respectively). The same was true for CMC counts at ∂S1-S0 (P=0.024).

No differences were found in CC counts according to TNM stage, histologic type (clear vs. non-clear cell), ISUP pathologic grade, PADUA score[25], R.E.N.A.L. score[26], and tumor size (Supplementary Material). Additionally, no significant correlation was found between CTC or CMC counts and tumor diameter, volume, parenchymal contact area, or attenuation value measured by CT scan.

At a median follow-up of 12.5 months, no difference was found in complication rates, PFS, or OS between groups (Supplementary Material). Subgroup analysis by CC type and count showed no survival difference either.

5.5 . DISCUSSION

This study suggests that an NT LRN does not reduce CTC or CMC release or impact survival. It also shows no increase in CC release with surgical manipulation. Similar findings by Haga *et al.* showed no difference in CTC counts after LRN, contrary to open RN (7.7 ± 6.8 to 22.5 ± 26.3, $p<0.05$)[28]. The study suggested that minimally invasive surgery reduced CC release. Conversely, two

studies reported an increased CTC detection rate after RCC surgery, but CTC counts were not studied[14].

In the present study, a progressive reduction in CMC counts was observed over time, most of which at D1. This was significant only in the C group, probably due to a random higher baseline count and small sample size. Similar findings of a progressive decrease in CTC counts during follow-up in M0 patients were reported by Wang *et al*[29].

CTC detection rates were low, possibly due to the predominance of localized low-stage tumors. Additionally, RCC typically has lower epithelial CTC counts due to a higher incidence of EMT compared to other tumor types, with loss of epithelial markers[5,6]. Although a progressive CTC decline was observed across time points, it was not significant, probably due to low baseline counts and small sample size.

The only clinicopathological parameter that correlated with total CC and CMC counts was MAP score, an imagiological surrogate marker of peri-renal inflammation. No other clinicopathologic or laboratory parameters correlated with CC counts and the same was observed for tumor diameter, volume, parenchymal contact area, or attenuation value.

The NT technique proved to be faster and as safe as the C technique. NT RN took on average 23 minutes less than the C technique, which is an advantage *per se* and may favor the choice of this technique.

The healthy control group confirmed no CCs. However, despite the absence of CTCs, a significant number of CMCs were identified in all patients of the chronic inflammation control group. The same was true for oncocytoma patients. None of these patients developed cancer during follow-up. Furthermore, CMC counts in these two groups did not differ from RCC patients. This was surprising because the literature reports the absence of CCs in controls using the same criteria and raises the question of the nature of CMCs[5]. The scientific community has been naming these cells as mesenchymal CTCs. To our knowledge, no study to date has included an inflammation control group. We may wonder if these cells are

CTCs or a subset of leukocytes with low CD45 expression, cancer-associated fibroblasts, or another inflammatory cell. They may even be different cell types. There is a well-studied relationship between cancer and inflammation that may help explain these findings[30]. The correlation between CMC counts and MAP scores further points in this direction. Nevertheless, the decrease in CMC counts after surgery suggests that they are mainly from tumor origin. Characterization of CCs with four markers is a limitation of most detection devices[5]. Caution should be exercised in classifying these cells as CTCs. Future downstream CMC analysis and improved biomarker selection are warranted to elucidate their true nature.

The main limitations of this study were its small sample size, low power to detect small differences in CC counts between groups and time points, and the short follow-up, hindering clinical outcome analysis. Additionally, CC downstream analysis was not performed.

Peri-operative CTC kinetics is still poorly understood, and its clinical significance remains unclear. This study suggests no advantage in early pedicle ligation. Has the last Robson principle fallen? Larger studies are crucial to confirm this hypothesis. Furthermore, a more comprehensive analysis of CCs and their interaction with the immune system may increase the understanding of RCC and improve future treatment protocols.

5.6. CONCLUSIONS

NT LRN did not reduce CC release or improve survival compared with C LRN. However, it proved to be faster and as safe as the conventional technique. CMCs were found in chronic inflammation and oncocytoma patients and decreased after surgery, suggesting tumor origin but questioning their CTC status.

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5.8. SUPPLEMENTARY MATERIAL

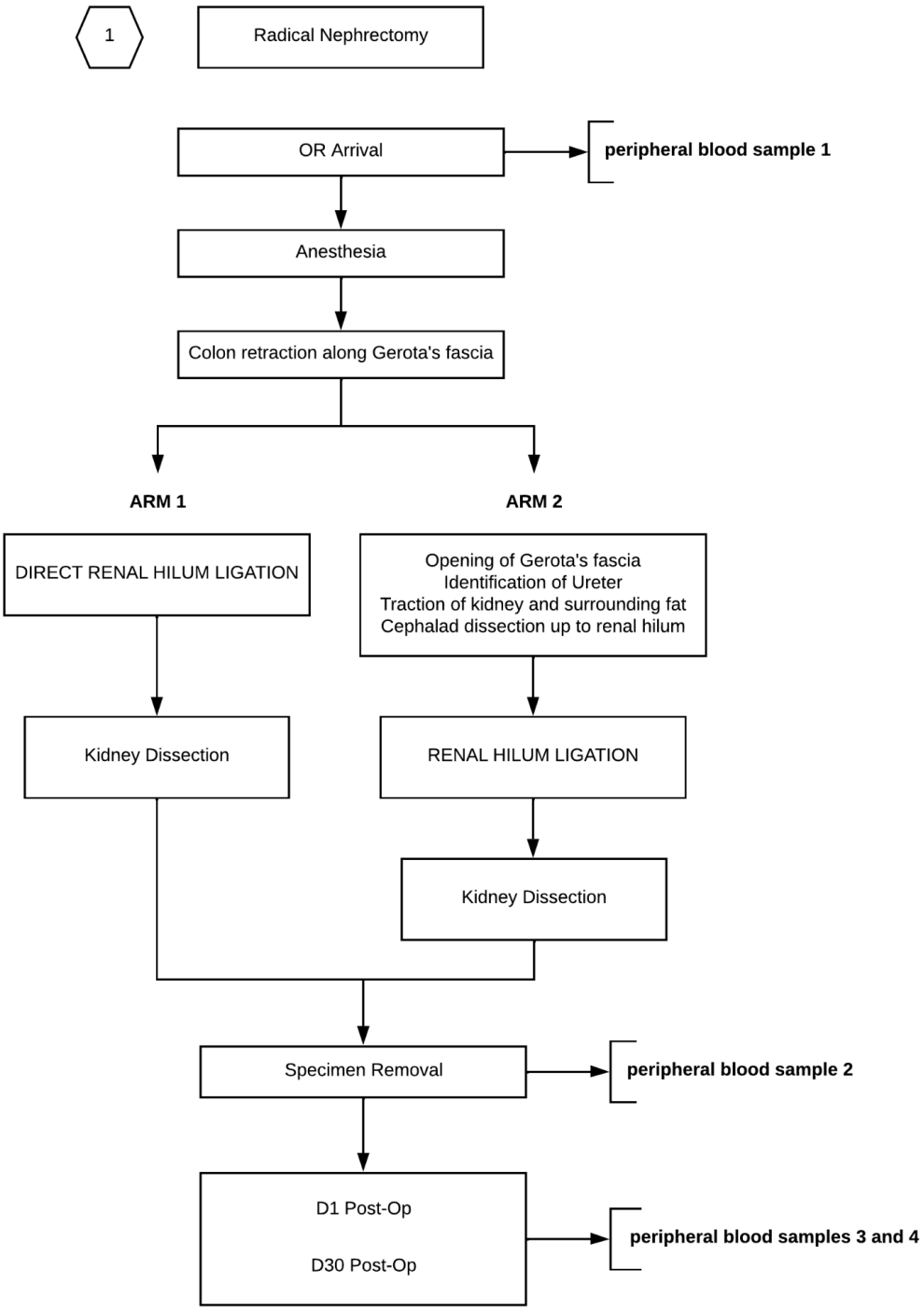


Figure S1. Study protocol flow chart.

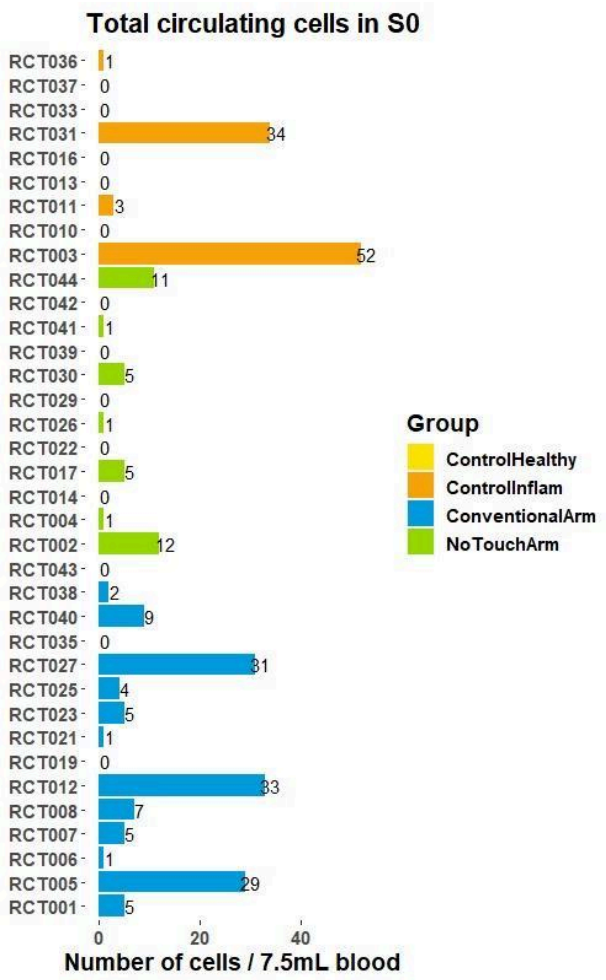
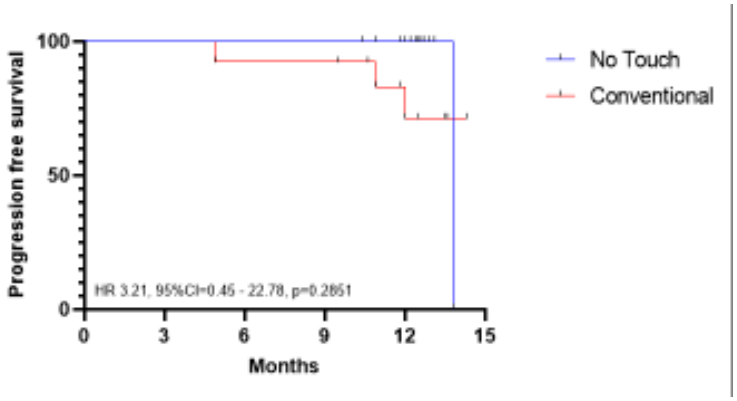
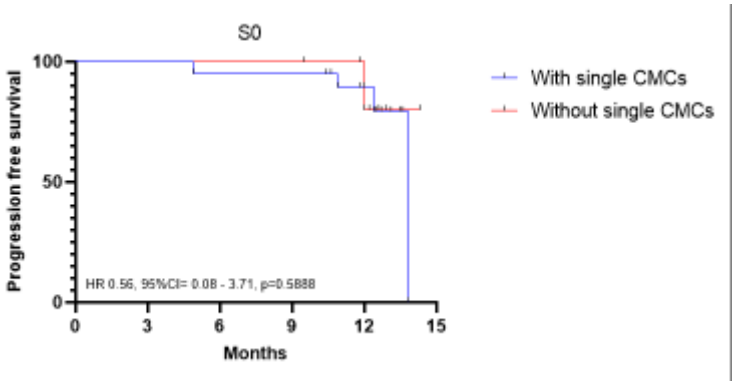
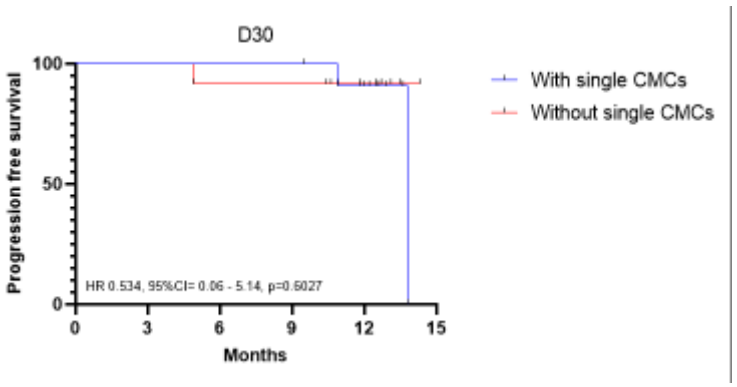


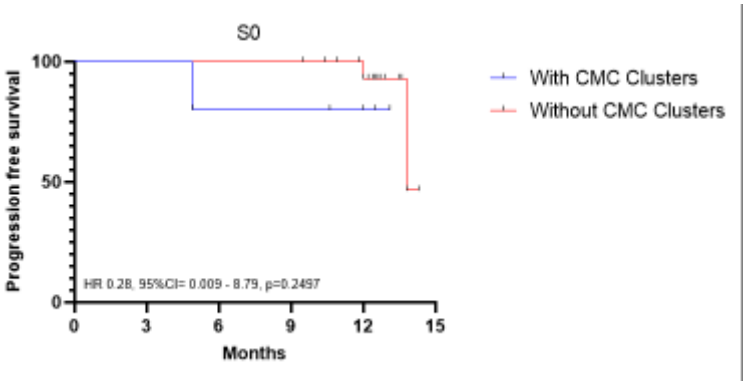
Figure S2. CMC counts per patient and time points.

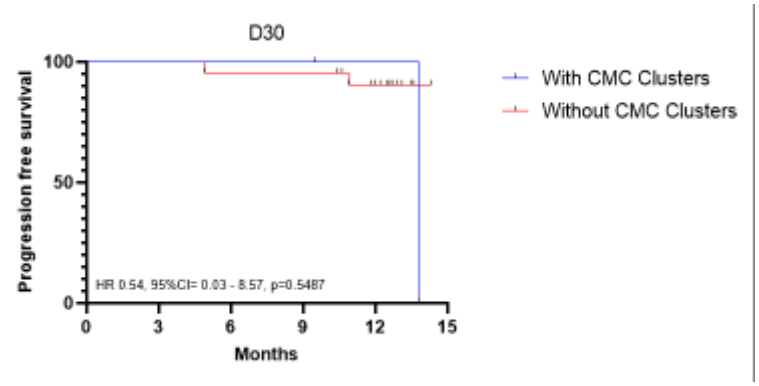
Figures S3. Survival (Kaplan-Meyer) Analysis

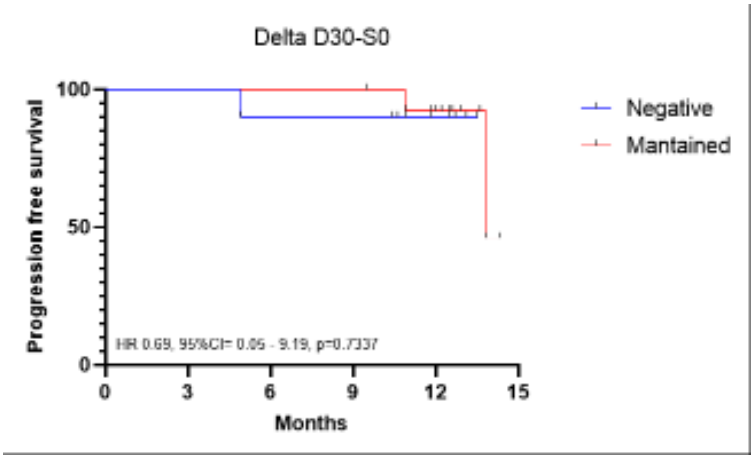


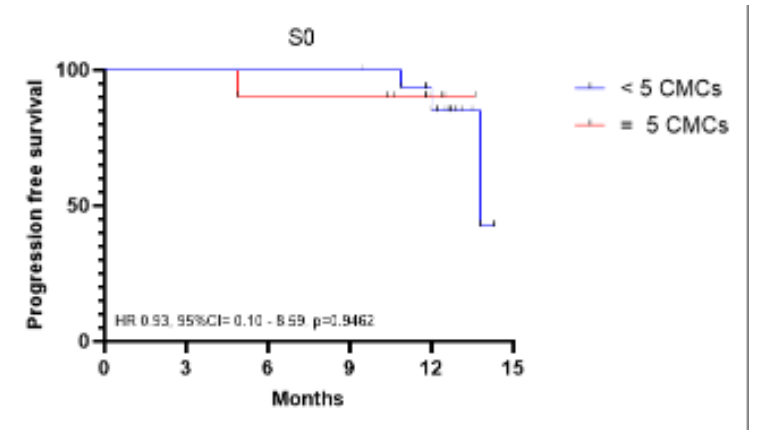


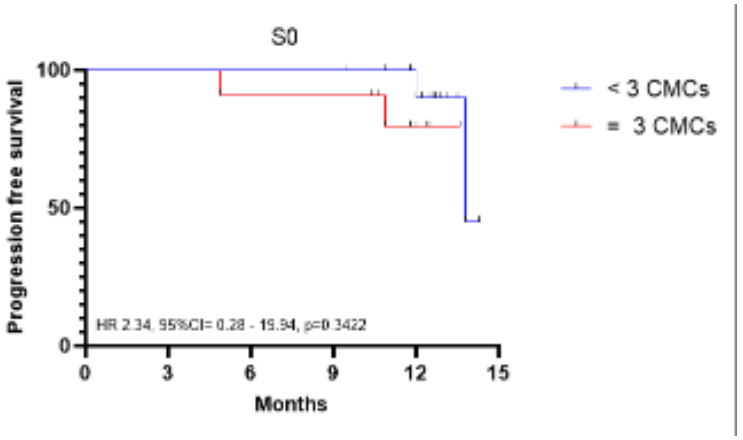


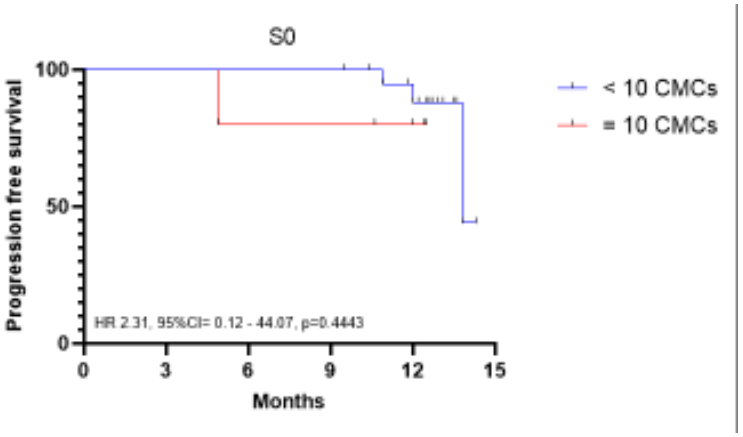


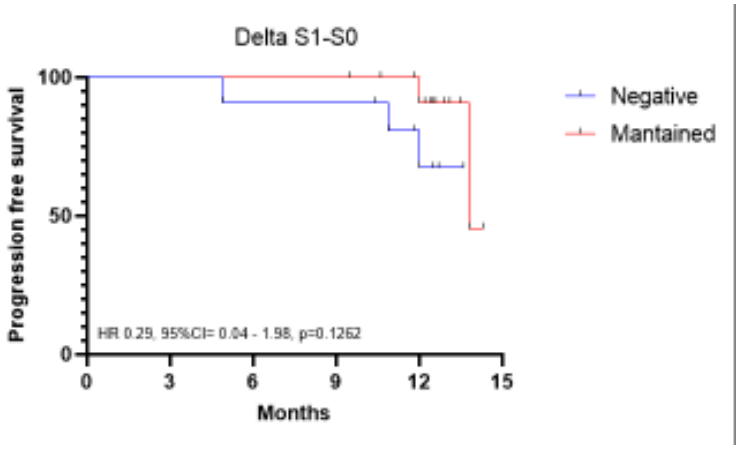

D30
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 0.54, 95%CI= 0.03 - 8.57, p=0.5487
With CMC Clusters
Without CMC Clusters


Delta D30-S0
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 0.69, 95%CI= 0.05 - 9.19, p=0.7337
Negative
Mantained


S0
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 0.93, 95%CI= 0.10 - 8.59, p=0.9462
< 5 CMCs
= 5 CMCs


S0
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 2.34, 95%CI= 0.28 - 19.94, p=0.3422
< 3 CMCs
= 3 CMCs


S0
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 2.31, 95%CI= 0.12 - 44.07, p=0.4443
< 10 CMCs
= 10 CMCs


Delta S1-S0
Progression free survival
100
50
0
0
3
6
9
12
15
Months
HR 0.29, 95%CI= 0.04 - 1.98, p=0.1262
Negative
Mantained

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT **2010 Flow Diagram**

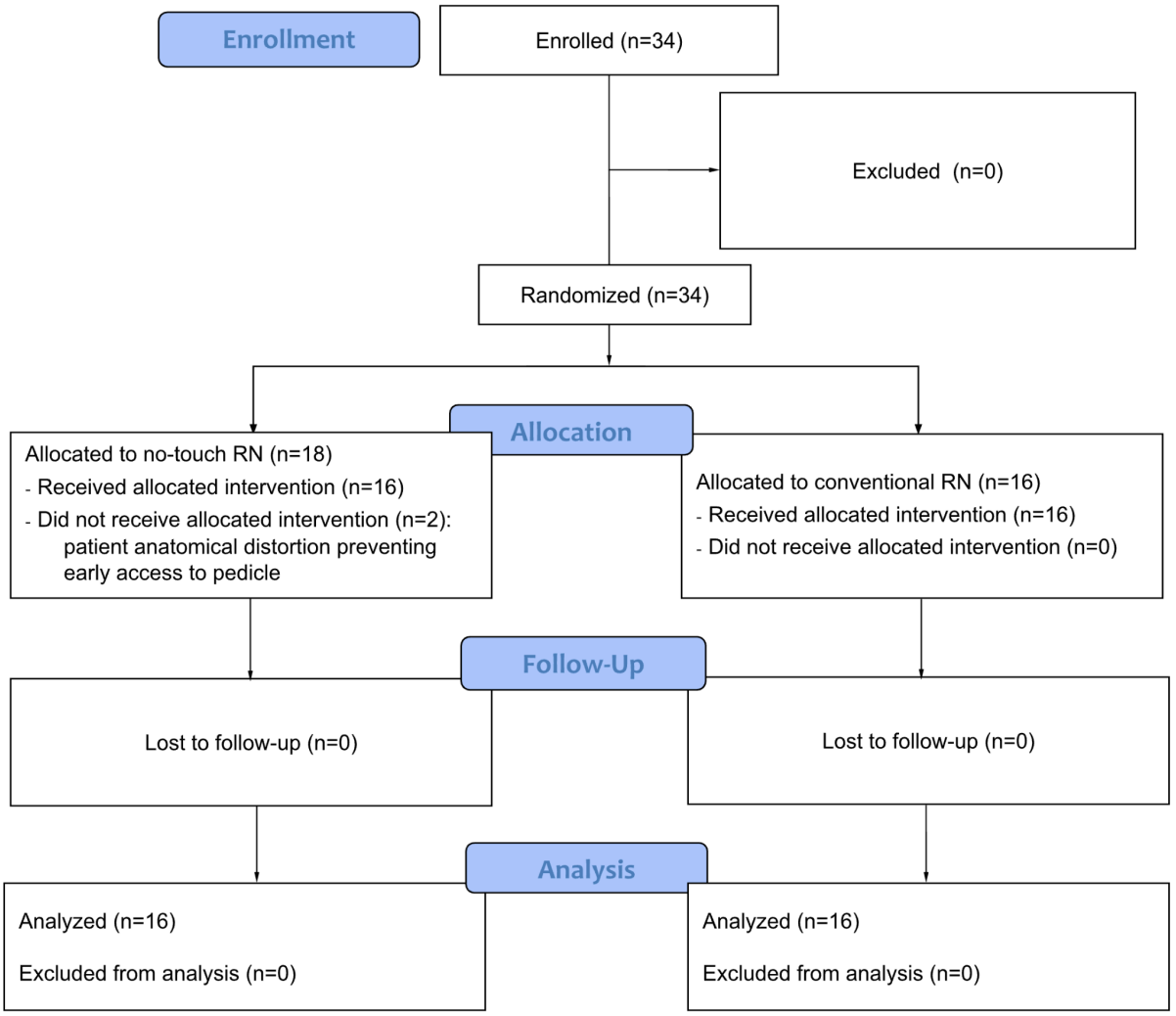


Figure S4. CONSORT 2010 Flow Diagram

Table S1. CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item Nº	Checklist item	Reported on page
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	3-4
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	5-6
	2b	Specific objectives or hypotheses	6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6-7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	10
Participants	4a	Eligibility criteria for participants	6-7
	4b	Settings and locations where the data were collected	6

Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7-8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	—
Sample size	7a	How sample size was determined	9
	7b	When applicable, explanation of any interim analyses and stopping guidelines	—
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	9
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	9
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	9
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	9
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	9
	11b	If relevant, description of the similarity of interventions	—

Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	9
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	10
	13b	For each group, losses and exclusions after randomisation, together with reasons	10
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6, 30
	14b	Why the trial ended or was stopped	—
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	11-15
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	21-22
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	27-30
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	29
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	29-20
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	—

Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	33
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	31-33
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	31-33
Other information			
Registration	23	Registration number and name of trial registry	8
Protocol	24	Where the full trial protocol can be accessed, if available	8
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	35

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

Table S2. CC count time point delta differences between RCC intervention groups

	S0 Vs S1			S0 Vs D1			S0 Vs D30			D1 Vs D30			S1 Vs D1			S1 Vs D30		
	NT	**C**	**O**	**NT**	**C**	**O**	**NT**	**C**	**O**	**NT**	**C**	**O**	**NT**	**C**	**O**	**NT**	**C**	**O**
Single CTCs	NA	1	NA	0.174	1	NA	1	1	NA	0.371	0.586	NA	0.174	1	NA	1	0.586	NA
Epithelial CTCs	NA	1	NA	0.174	1	NA	1	1	NA	0.371	0.586	NA	0.174	1	NA	1	0.586	NA
EMT CTCs	NA	NA	NA	1.000	NA	NA	NA	NA	NA	1	NA	NA	1.000	NA	NA	NA	NA	NA
CMCs	0.518	0.753	0.625	1	**0.049**	0.125	0.546	**0.029**	0.125	0.590	0.777	0.181	0.753	**0.009**	0.125	0.915	**0.034**	0.269
Single CMCs	0.944	1	0.098	1	**0.049**	0.125	0.328	**0.023**	0.125	0.343	0.723	0.345	0.753	**0.009**	0.125	0.410	**0.013**	0.345
CMCs in clusters	0.371	0.855	0.789	0.371	0.181	0.371	1	0.361	0.371	1	1	1	NA	1	0	1	1	1
Clusters	0.346	0.713	0.586	0.346	0.181	0.371	1	0.269	0.371	1	1	1	NA	1	0	1	1	1
Total circulating cells	0.518	0.609	0.625	0.830	**0.025**	0.125	0.546	**0.030**	0.125	0.462	0.830	0.181	0.571	**0.008**	0.125	1.000	0.058	0.269
Single circulating cells	0.944	0.842	0.098	0.830	**0.025**	0.125	0.328	**0.025**	0.125	0.292	0.886	0.181	0.571	**0.008**	0.125	0.590	**0.017**	0.345

p values with Wilcoxon

Table S3. CC count relative time point delta differences between RCC intervention groups and negativation rates

	Diferença das variações relativas médias S1 - S0		Diferença das variações relativas médias D30 - S0		Negativation Rate S1 - S0 n=27			Negativation Rate D30 - S0 n=25		
	NT	**C**	**NT**	**C**	**NT**	**C**	**p value**	**NT**	**C**	**p value**
Single CTCs	0.00	-0.26	NA	0.09	NA	NA	NA	0	0.04	NA
Epithelial CTCs	0.00	-0.26	NA	0.09	NA	NA	NA	0	0.04	NA
EMT CTCs	0.00	0.00	NA	NA	NA	NA	NA	NA	NA	NA
CMCs	-33.33%	-17.36%	-69.67%	-81.46%	14.81%	7.41%	0.141	20.00%	20.00%	0.304
Single CMCs	-22.48%	-12.92%	-82.56%	-83.19%	14.81%	7.41%	0.141	20.00%	20.00%	0.304
CMCs in clusters	-100.00%	-42.52%	7.14%	-71.89%	7.41%	11.11%	1	8.00%	12.00%	NA
Clusters	-100.00%	-50.00%	5.88%	-82.50%	7.41%	11.11%	1	8.00%	12.00%	NA
Total circulating cells	-33.33%	NA	-66.67%	NA	14.81%	7.41%	0.129	16.00%	24.00%	0.638
Single circulating cells	-22.48%	-15.94%	-78.68%	-79.15%	14.81%	7.41%	0.129	16.00%	24.00%	0.638

§ p values for Fisher test

Table S4.1. CC counts for the whole intervention group according to time point

	S0	S1	D1	D30	Mean of cell count difference S1 - S0	Mean of cell count difference D30 - S0
	All cohort	All cohort	All cohort	All cohort	All cohort	All cohort
Single CTCs	0.19	0.04	0.64	0.24	-0.15	0.05
Epithelial CTCs	0.19	0.04	0.2	0.24	-0.15	0.05
EMT CTCs	0	0	0.44	0	0	0
CMCs	6.04	4.78	1.64	1.28	-1.26	-4.76
Single CMCs	5.15	4.37	1.64	0.88	-0.78	-4.27
CMCs in clusters	0.89	0.41	0	0.4	-0.48	-0.49
Clusters	3	0.11	0	0.12	-2.89	-2.88
Total circulating cells	6.22	4.82	2.28	1.52	-1.4	-4.7
Single circulating cells	5.33	4.41	2.28	1.12	-0.92	-4.21

values are means

Table S4.2. CC count differences between timepoints in the whole intervention group

	S0 Vs S1	S0 Vs D1	S0 Vs D30	D1 Vs D30	S1 Vs D1	S1 Vs D30
	p	*p*	*p*	*p*	*p*	*p*
Single CTCs	1	0.410	0.854	0.784	0.203	0.345
Epithelial CTCs	1	0.583	0.854	1	0.203	0.345
EMT CTCs	NA	1.000	NA	1	1	NA
CMCs	0.547	0.096	**0.016**	0.504	**0.035**	**0.042**
Single CMCs	0.935	0.104	**0.006**	0.342	**0.035**	**0.006**
CMCs in clusters	0.400	0.058	0.445	0.346	1	1
Clusters	0.339	0.055	0.343	0.371	1	1
Total circulating cells	0.437	0.074	**0.024**	0.679	**0.049**	0.078
Single circulating cells	0.777	0.087	**0.010**	0.545	0.054	**0.010**

p values - Wilcoxon

Table S4.3. CC count differences between clear cell and non-clear cell RCC

	Clear Cell Vs Non-Clear cell
	S0
Single CTCs	0.192
Epithelial CTCs	0.192
EMT CTCs	0.959
CMCs	0.878
Single CMCs	0.250
CMCs in clusters	0.236
Clusters	0.645
Total circulating cells	0.798
Single circulating cells	0.798

Table S4.4. CC count differences between RCC intervention groups according to time between renal vein exposure and ligation

	Time between renal vein exposure and ligation (min)*	
	NT	**CG**
Single CTCs	NA	0.5067 (r=0.186)
Epithelial CTCs	NA	0.5067 (r=0.186)
EMT CTCs	NA	NA
CMCs	0.9088 (r=0.037)	0.0268 (r=-0.569)
Single CMCs	1 (r=0)	0.0299 (r=-0.560)
CMCs in clusters	0.5876 (r=0.174)	0.4843 (r=-0.196)
Clusters	0.5392 (r=0.197)	0.4843 (r=-0.196)
Total circulating cells	0.9088 (r=0.037)	0.0512 (r=-0.512)
Single circulating cells	1 (r=0)	0.0561 (r=-0.503)

*Spearman's correlation; p-value (r coefficient)

Table S5.1 Correlation between CC counts at S0 and clinicopathological variables — no-touch arm (n=12)

	Single CTCs		Single CMC		Clusters		Total CMC		Total CCs	
	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*
Age at surgery (yr)	NA	NA	0.184	0.5671	0.391	0.2083	0.232	0.4677	0.232	0.4677
BMI (kg/m2)	NA	NA	-0.073	0.8212	-0.097	0.7635	-0.062	0.8474	-0.062	0.8474
Weight (kg)	NA	NA	0.184	0.5664	0.163	0.6119	0.209	0.5152	0.209	0.5152
Height (cm)	NA	NA	0.384	0.2175	0.162	0.6145	0.361	0.2487	0.361	0.2487
GFR (mL/min/1.73)	NA	NA	-0.248	0.4363	0	1	-0.187	0.5613	-0.187	0.5613
CKD	NA	NA	0.242	0.4491	-0.099	0.7597	0.168	0.6025	0.168	0.6025
Tumor size (max. diam., mm)	NA	NA	-0.57	0.0531	-0.13	0.6882	-0.56	0.0583	-0.56	0.0583
Operative time (min)	NA	NA	-0.293	0.3558	-0.097	0.7635	-0.302	0.3393	-0.302	0.3393
Blood loss (ml)	NA	NA	-0.192	0.55072	-0.49	0.10576	-0.246	0.44173	-0.246	0.44173
Time until renal vein exposure (min)	NA	NA	-0.444	0.14782	-0.228	0.4762	-0.456	0.136	-0.456	0.136
Time between renal vein exposure and ligation (min)	NA	NA	0	1	0.197	0.53921	0.037	0.90881	0.037	0.90881
Tumor maximum diameter (mm)	NA	NA	-0.446	0.14648	0	1	-0.425	0.16891	-0.425	0.16891
Hb (g/dL)	NA	NA	-0.117	0.71702	-0.097	0.76347	-0.088	0.78566	-0.088	0.78566
Leucocyte Count (x10^9/L)	NA	NA	-0.402	0.19536	-0.259	0.41611	-0.421	0.17301	-0.421	0.17301
Neutrophil Count (x10^9/L)	NA	NA	-0.337	0.28458	-0.195	0.54433	-0.356	0.25655	-0.356	0.25655
Lymphocyte Count (x10^9/L)	NA	NA	-0.307	0.33196	-0.389	0.21182	-0.359	0.25222	-0.359	0.25222
Neutrophil to Lymphocyte Ratio	NA	NA	-0.132	0.6837	0.324	0.30443	-0.059	0.85654	-0.059	0.85654
Platelet Count (x10^9/L)	NA	NA	**-0.614**	**0.03378**	-0.065	0.84148	**-0.608**	**0.03612**	**-0.608**	**0.03612**
Creatinine (mg/dL)	NA	NA	0.0658	0.83911	-0.1296	0.68821	-0.0037	0.99099	-0.0037	0.99099
GFR (mL/min/1.73)	NA	NA	-0.248	0.43626	0	1	-0.187	0.56131	-0.187	0.56131

Urea (mg/dL)	NA	NA	0.27	0.39544	-0.13	0.68821	0.198	0.53806	0.198	0.53806
Calcium (mg/dL)	NA	NA	0.177	0.60213	-0.411	0.20936	0.082	0.8114	0.082	0.8114
INR	NA	NA	-0.135	0.67479	-0.519	0.08372	-0.216	0.49948	-0.216	0.49948
Albumin (g/dL)	NA	NA	0.06	0.86086	-0.187	0.58153	-0.007	0.98319	-0.007	0.98319
CRP (mg/L)	NA	NA	-0.504	0.11384	-0.374	0.25665	-0.549	0.08047	-0.549	0.08047

Table S5.2 Correlation between CC counts at S0 and clinicopathological variables — conventional (n=15)

	Single CTCs		Single CMC		Clusters		Total CMC		Total CCs	
	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*
Age at surgery (yr)	-0.372	0.172	-0.077	0.785	-0.267	0.336	-0.095	0.736	-0.259	0.352
BMI (kg/m2)	0.062	0.827	-0.397	0.143	-0.171	0.542	-0.371	0.173	-0.352	0.198
Weight (kg)	0.093	0.742	-0.470	0.077	-0.084	0.765	-0.437	0.103	-0.407	0.132
Height (cm)	0.124	0.660	-0.219	0.433	0.081	0.775	-0.186	0.506	-0.146	0.603
GFR (mL/min/1.73)	-0.372	0.172	0.212	0.448	-0.069	0.807	0.183	0.514	0.043	0.878
CKD	0.359	0.189	-0.181	0.519	0.105	0.710	-0.143	0.612	-0.013	0.963
Tumor size (max. diam., mm)	-0.062	0.827	0.031	0.914	-0.171	0.542	0.016	0.954	0.000	1.000
Operative time (min)	0.310	0.261	-0.445	0.097	-0.068	0.810	-0.436	0.104	-0.319	0.246
Blood loss (ml)	-0.310	0.260	-0.188	0.501	-0.228	0.414	-0.178	0.527	-0.307	0.265
Time until renal vein exposure (min)	0.310	0.262	-0.267	0.335	0.156	0.579	-0.271	0.329	-0.162	0.564
Time between renal vein exposure and ligation (min)	0.186	0.507	-0.560	0.030	-0.196	0.484	-0.569	0.027	-0.512	0.051
Tumor maximum diameter (mm)	-0.125	0.657	-0.144	0.609	-0.297	0.283	-0.139	0.622	-0.196	0.483
Hb (g/dL)	-0.186	0.507	-0.398	0.142	-0.581	0.023	-0.405	0.134	-0.491	0.063
Leucocyte Count (x10^9/L)	0.310	0.262	-0.190	0.498	-0.377	0.166	-0.195	0.486	-0.068	0.808
Neutrophil Count (x10^9/L)	0.309	0.262	-0.464	0.082	-0.396	0.144	-0.442	0.099	-0.342	0.212
Lymphocyte Count (x10^9/L)	-0.186	0.508	0.486	0.067	0.046	0.871	0.446	0.096	0.409	0.130
Neutrophil to Lymphocyte Ratio	0.247	0.374	-0.628	0.012	-0.204	0.465	-0.592	0.020	-0.533	0.041
Platelet Count (x10^9/L)	0.062	0.827	0.164	0.560	-0.178	0.527	0.147	0.600	0.155	0.581
Creatinine (mg/dL)	0.371	0.173	-0.503	0.056	-0.013	0.964	-0.463	0.082	-0.344	0.209
GFR (mL/min/1.73)	-0.372	0.172	0.212	0.448	-0.069	0.806	0.183	0.514	0.043	0.878

Urea (mg/dL)	0.371	0.173	-0.478	0.072	-0.167	0.551	-0.440	0.101	-0.315	0.252
Calcium (mg/dL)	-0.345	0.227	-0.089	0.763	-0.270	0.351	-0.080	0.786	-0.260	0.370
INR	-0.311	0.279	0.111	0.705	0.378	0.183	0.151	0.606	0.016	0.958
Albumin (g/dL)	-0.139	0.635	0.016	0.958	-0.263	0.363	-0.016	0.958	-0.074	0.802
CRP (mg/L)	0.447	0.109	-0.268	0.355	0.034	0.909	-0.246	0.397	-0.080	0.787

Table S5.3 Correlation between CC counts at S0 and clinicopathological variables — controls (n=9)

	Single CTCs		Single CMC		Clusters		Total CMC		Total CCs	
	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*
Age at surgery (yr)	NA	NA	-0.329	0.388	-0.365	0.334	-0.329	0.388	-0.329	0.388
BMI (kg/m2)	NA	NA	1.000	0.333	0.866	0.333	1.000	0.333	1.000	0.333
Weight (kg)	NA	NA	0.500	1.000	0.866	0.333	0.500	1.000	0.500	1.000
Height (cm)	NA	NA	0.500	1.000	0.866	0.333	0.500	1.000	0.500	1.000
GFR (mL/min/1.73)	NA	NA	0.529	0.143	0.593	0.092	0.529	0.143	0.529	0.143
CKD	NA	NA	-0.551	0.125	-0.641	0.063	-0.551	0.125	-0.551	0.125
Tumor size (max. diam., mm)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Operative time (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Blood loss (ml)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Time until renal vein exposure (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Time between renal vein exposure and ligation (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tumor maximum diameter (mm)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hb (g/dL)	NA	NA	-0.018	0.963	-0.365	0.334	-0.018	0.963	-0.018	0.963
Leucocyte Count (x10^9/L)	NA	NA	0.256	0.507	0.068	0.861	0.256	0.507	0.256	0.507
Neutrophil Count (x10^9/L)	NA	NA	0.000	1.000	0.160	0.681	0.000	1.000	0.000	1.000
Lymphocyte Count (x10^9/L)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Neutrophil to Lymphocyte Ratio	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Platelet Count (x10^9/L)	NA	NA	-0.292	0.446	-0.342	0.367	-0.292	0.446	-0.292	0.446
Creatinine (mg/dL)	NA	NA	-0.511	0.160	-0.593	0.092	-0.511	0.160	-0.511	0.160
GFR (mL/min/1.73)	NA	NA	0.529	0.143	0.593	0.092	0.529	0.143	0.529	0.143

Urea (mg/dL)	NA	NA	0.028	0.944	-0.115	0.769	0.028	0.944	0.028	0.944
Calcium (mg/dL)	NA	NA	0.236	0.610	0.045	0.924	0.236	0.610	0.236	0.610
INR	NA	NA	-0.275	0.474	0.011	0.977	-0.275	0.474	-0.275	0.474
Albumin (g/dL)	NA	NA	-0.019	0.964	-0.063	0.883	-0.019	0.964	-0.019	0.964
CRP (mg/L)	NA	NA	0.091	0.815	-0.091	0.815	0.091	0.815	0.091	0.815

Table S5.4 Correlation between CC counts at S0 and clinicopathological variables — inflammatory control (n=4)

	Single CTCs		Single CMC		Clusters		Total CMC		Total CCs	
	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*
Age at surgery (yr)	NA	NA	-0.400	0.750	-0.211	0.789	-0.400	0.750	-0.400	0.750
BMI (kg/m2)	NA	NA		NA		NA		NA		NA
Weight (kg)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Height (cm)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
GFR (mL/min/1.73)	NA	NA	0.800	0.333	0.738	0.262	0.800	0.333	0.800	0.333
CKD	NA	NA	-0.949	0.051	-0.889	0.111	-0.949	0.051	-0.949	0.051
Tumor size (max. diam., mm)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Operative time (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Blood loss (ml)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Time until renal vein exposure (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Time between renal vein exposure and ligation (min)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Tumor maximum diameter (mm)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Hb (g/dL)	NA	NA	-0.600	0.417	-0.738	0.262	-0.600	0.417	-0.600	0.417
Leucocyte Count (x10^9/L)	NA	NA	0.400	0.750	0.316	0.684	0.400	0.750	0.400	0.750
Neutrophil Count (x10^9/L)	NA	NA	0.800	0.333	0.632	0.368	0.800	0.333	0.800	0.333
Lymphocyte Count (x10^9/L)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Neutrophil to Lymphocyte Ratio	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Platelet Count (x10^9/L)	NA	NA	-0.800	0.333	-0.632	0.368	-0.800	0.333	-0.800	0.333
Creatinine (mg/dL)	NA	NA	-0.600	0.417	-0.738	0.262	-0.600	0.417	-0.600	0.417
GFR (mL/min/1.73)	NA	NA	0.800	0.333	0.738	0.262	0.800	0.333	0.800	0.333

Urea (mg/dL)	NA	NA	0.000	1.000	-0.211	0.789	0.000	1.000	0.000	1.000
Calcium (mg/dL)	NA	NA	-0.500	1.000	-0.500	1.000	-0.500	1.000	-0.500	1.000
INR	NA	NA	0.000	1.000	0.211	0.789	0.000	1.000	0.000	1.000
Albumin (g/dL)	NA	NA	-0.400	0.750	-0.211	0.789	-0.400	0.750	-0.400	0.750
CRP (mg/L)	NA	NA	-0.400	0.750	-0.316	0.684	-0.400	0.750	-0.400	0.750

Table S6. Correlation between CC counts and clinicopathological variables at S0 and S1-S0 delta

	S0							**CTCn difference at end of surgery (∂CTC) (S1-S0)**						
	Single CTCs	**Total CMC**	Single CMCs	CMCs in clusters	Clusters	**Total CCs**	Single CCs	**Single CTCs**	**Total CMC**	Single CMCs	CMCs in clusters	Clusters	**Total CCs**	Single CCs
Clear cell vs non-clear cell	0.220	0.980	0.898	0.251	0.266	0.664	0.818	0.220	0.650	0.820	0.581	0.605	0.513	0.980
Microvascular invasion: 0,1	0.7029	0.774	0.823	1.000	1.000	0.633	0.680	0.703	0.851	0.638	0.932	0.898	0.925	0.707
Renal vein (segmental) invasion, n (%)	0.755	0.861	0.917	0.880	0.840	0.754	0.808	0.755	0.891	0.945	0.778	0.743	0.837	0.891
pT: 1,2,3	0.240	0.223	0.254	0.904	0.913	0.495	0.567	0.240	0.694	0.869	0.960	0.980	0.863	0.962
Exophytic rate: 1, 2, 3	0.584	0.208	0.276	0.124	0.106	0.282	0.369	0.535	0.324	0.362	0.228	0.197	0.354	0.350
Renal sinus: 0, 1	0.656	0.743	0.788	0.212	0.212	0.613	0.656	0.656	0.838	0.837	0.357	0.356	0.748	0.930
Total PADUA score: 6-13	0.333	0.065	0.063	0.188	0.187	0.159	0.163	0.333	0.281	0.526	0.126	0.125	0.335	0.537
PADUA risk category: High, Intermediate, Low	0.810	0.630	0.633	0.396	0.395	0.615	0.623	0.810	0.576	0.766	0.159	0.159	0.564	0.715
Total R.E.N.A.L score (4-11)	0.722	0.846	0.798	0.505	0.513	0.832	0.807	0.722	0.630	0.321	0.240	0.243	0.725	0.435
R.E.N.A.L.: High, Intermediate, Low	0.535	0.388	0.318	0.821	0.807	0.471	0.393	0.535	0.792	0.864	0.939	0.938	0.874	0.943
MAP score: 0-5	0.493	0.055	0.054	0.116	0.110	**0.031**	**0.033**	0.493	**0.024**	0.059	0.113	0.108	**0.020**	0.055

p-value calculated with Kruskal-Wallis rank sum test

Table S7. Correlation coefficients for the relation between CC counts and CT imaging variables

			PP absolute washout	PP relative washout	LP absolute washout	LP relative washout	Tumor largest diameter (mm)	Tumor Volume (cm3)	Tumour parenchyma contact area (cm3)	Attenuation Value arterial/corticomedular (25-45seg) (HU)
All cohort (S0) n=27	Single CTCs	r2	-0.252	-0.151	-0.277	-0.201	-0.050	-0.050	-0.076	-0.252
		p	0.205	0.452	0.162	0.314	0.803	0.803	0.708	0.2051
	Single CMC	r2	-0.111	-0.131	-0.201	-0.144	-0.254	-0.254	-0.074	0.105
		p	0.583	0.516	0.315	0.475	0.202	0.202	0.714	0.6014
	CMC Clusters	r2	-0.125	-0.102	-0.160	-0.192	-0.104	-0.104	0.022	-0.042
		p	0.536	0.613	0.425	0.337	0.606	0.606	0.913	0.8354
	Total CMC	r2	-0.126	-0.142	-0.213	-0.158	-0.240	-0.240	-0.063	0.092
		p	0.531	0.480	0.287	0.430	0.227	0.227	0.756	0.648
	Total circulating cells	r2	-0.197	-0.177	-0.301	-0.219	-0.259	-0.259	-0.089	0.019
		p	0.326	0.378	0.128	0.273	0.193	0.193	0.658	0.9239
All cohort (S1) n=27	Single CTCs	r2	-0.252	0.180	-0.277	-0.201	-0.050	-0.050	-0.076	-0.252
		p	0.205	0.452	0.162	0.314	0.803	0.803	0.708	0.2051
	Single CMC	r2	-0.228	-0.240	-0.196	-0.182	-0.057	-0.057	0.071	-0.125
		p	0.252	0.227	0.328	0.363	0.778	0.778	0.725	0.533
	CMC Clusters	r2	-0.101	-0.076	-0.101	-0.101	-0.290	-0.290	-0.327	-0.126
		p	0.617	0.708	0.617	0.617	0.142	0.142	0.096	0.5314

	Total CMC	r2	-0.223	-0.234	-0.190	-0.177	-0.061	-0.061	0.070	-0.122
		p	0.265	0.240	0.342	0.378	0.764	0.764	0.727	0.5455
	Total circulating cells	r2	-0.266	-0.264	-0.236	-0.212	-0.061	-0.061	0.067	-0.166
		p	0.180	0.183	-0.236	0.288	0.761	0.761	0.739	0.4092

p - p-value; r2 - squared Spearman's correlation coeficient; PP - parenchymal phase; LP - late phase; Parenchymal phase absolute washout [(B-C)/(B-A)]; Parenchymal phase relative washout [(B-C)/B]; Late phase absolute washout [(B-D)/(B-A)]; Late phase relative washout [(B-D)/B]

6. OVERALL DISCUSSION

6.1. GLOBAL PROJECT DISCUSSION

CTCs have been improving our understanding of cancer biology and have shown great potential to become relevant diagnostic, prognostic, and treatment-monitoring biomarkers. However, there is currently not enough evidence base to warrant clinical applications in RCC. In this research project, we aimed to add some data to the current knowledge base on the role of CTCs in RCC.

The first step was to conduct a systematic review of the literature on the topic. Although there were some systematic reviews on the role of CTCs in some cancers like melanoma[1], colorectal cancer[2], and pancreatic cancer[3], there were none on RCC. Hence, to our knowledge, ours was the first systematic review concerning the use of CTCs in RCC patients. It identified studies with many limitations, generally with a high risk of bias and a low level of evidence. No prospective randomized trials were found and most studies were of an exploratory nature. Most study designs found were observational and only a few were cohort studies, with small and unbalanced samples. A wide variety of enrichment and detection techniques have been used so far, and also a variety of molecular markers. This fact hinders comparisons between studies and conclusive statements or conclusions on the matter.

Having systematically reviewed the literature, the technology that seemed more promising and with better efficiency was microfluidics. Hence, we selected a microfluidics platform for the next steps of the research project. Having partnered with INL and Ruby Nanomed, we advanced to the in vitro and clinical validation of the microfluidic size-based CTC detection device RUBYchip™.

After having successfully validated the RUBYchip™ for RCC patients, we moved on to using it in the RCT we had designed to determine if CTC release could be reduced during RN using a no-touch technique.

In the following paragraphs, we will make an integrated discussion of the findings of the three studies that constitute this Thesis, organized by the specific topics addressed.

6.2. CTC DETECTION TECHNIQUES

We classified CTC enrichment techniques into four categories: antibody-based (immunomagnetic beads or microfluidics), density-based, size-based (microfluidics and membrane filters), and electrophoresis-based. CTC detection and identification techniques were divided into five categories: immunocytochemistry, RT-PCR, cytomorphological criteria, flow cytometry with immunofluorescence, and FISH.

6.3. CIRCULATING CELL (CC) DETECTION RATES

Published results on CTC detection rates varied widely and were inconsistent between platforms and markers. This variety in results is not completely explained by differences in the clinical characteristics of the patient cohorts and was probably associated with the technical limitations and the heterogeneity and complexity of the multi-step sample processing procedures, prone to cell loss and lack of sensitivity[4]. Furthermore, cancer multi-clonality and EMT can change the expression of molecular markers, a phenomenon very frequent in RCC[5,6]. For this reason, many studies use a combination of markers, including mesenchymal markers, the most used of which is vimentin.

CellSearch® was considered the reference standard for many epithelial cancers for many years, due to its unique FDA-approved status, but not for RCC[7]. Initial RCC studies targeting EpCAM showed dismally low detection rates. The finding only 18.6% of RCC CTCs express EPCAM sparked different approaches to RCC cTC detection[8]. More recent studies showed 100% detection rates using size-based enrichment followed by FISH or flow cytometry immunofluorescence for CK and EpCAM[9–11]. This stresses the importance of molecular markers and detection techniques optimization to maximize the assays' sensitivity. In 2022, the Parsortix® microfluidic platform also received FDA approval for metastatic breast cancer CTC detection, which confirms microfluidics as a promising technology.

In our RUBYchip™ validation study, the mean capture efficiency in the spiking experiments with RCC cell culture lines was 74.9%. This rate was obtained at an optimal flow rate of 80 µL/min. This optimal rate was found after testing the device for multiple rates and RCC cell culture lines. This high efficiency was probably due to the absence of blood sample pre-processing, and to the chip's proprietary design, enhancing WBC clearance and preventing microclot formation[12]. The geometry of the poles inside the microfilters allows a delicate balance between allowing passage to smaller, more deformable blood cells, like erythrocytes and leukocytes, and the entrapment of larger less deformable cells, like CTCs. CTCs are known to be larger and with a more rigid cellular structure due to their large nuclei, and high cytoplasm-to-nucleus ratio[13]. However, there is always a wide variability of sizes and phenotypes[14]. This is why we tested the devices with three RCC cell culture lines. The RUBYchip™ revealed consistent detection rates across the three cell culture lines.

In the clinical validation phase, the RUBYchip™'s overall CC detection rate was 77.8%. We detected CCs in 80% of M1 patients and 75% of M0 patients. This is a higher detection rate than the overall median of 57% (IQR 55%) found in our systematic review[15]. Current CTC detection platforms tend to have lower sensitivity in RCC compared to other epithelial cancers, which is thought to be due to the loss of the epithelial markers normally used to isolate these cells, through the process of EMT[15]. The similarity in detection rates found in our validation study between localized and metastatic disease was an interesting finding, showing that most cancer patients have CTCs, even in early localized stages. What seems to vary with disease stage is the CTC counts and not the proportion of patients with CTCs[15].

On our RCT, the total CC detection rate was 75.0% at baseline. This was very similar to the validation phase detection rates. Epithelial CTCs were detected in 50% of the validation phase patients but in only in 3.7% of the RCT patient cohort. This very low CTC detection rate may be explained by the fact that tumors were localized and of low stage in this cohort.

In the validation study, the majority of the CCs in the M1TN groups were also CMCs and only one M1 patient presented epithelial CTCs. The CMC detection rate in the validation study was 38.9% overall, whereas in the RCT was 70.5%.

This is compatible with the abundant EMT process known to happen in advanced RCC. Although the metastatic process is not yet fully understood, EMT is known to play a role in CTC release, tumor progression, and treatment resistance[16]. An association between EMT and both treatment response and survival outcomes has been made in some cancer types[17,18].

6.4. CC COUNTS

In the clinical validation study, we found that M1 treatment-naive (M1TN) patients had significantly higher CTC and CMC counts than M1 patients under systemic treatment and progressing (M1TP). M1TN patients had 31.8 times more total CCs than M1TP patients ($p=0.0003$) and 15.9 more than M0 patients. These differences were mainly due to CMCs. M1TN patients presented 488 times more CMCs than M1TP patients ($p<0.0001$) and 31 times more than M0 patients ($p=0.007$).

These findings were in line with the literature. A study also found higher CTC counts in metastatic RCC patients compared to localized RCC ones (9.6 vs. 5.3 CTC/7.5 mL, respectively)[19]. Another study found that CTC counts were 2.2 times higher in late-stage (stages 3 and 4) compared to early-stage (stages 1 and 2) disease ($p<0.001$)[8]. These last authors also correlated mesenchymal CTCs with the RCC stage.

Epithelial CTC counts were very similar between the validation groups, suggesting that the disease stage did not affect this parameter. Interestingly we found no EMT CTCs in the entire cohort. This might have been due to low tumor stages in the M0 group, with few cancer cells yet in the process of EMT, and to a very advanced disease of M1 patients, where all cells had undergone full epithelial marker loss and mesenchymal marker gain.

Surprisingly, M1TP patients did not have a significant difference in CTC counts compared to M0 patients. This may be an indication systemic therapies are controlling the disease and keeping CC release in check.

6.5. CMC CLUSTERS

CTC clusters can be defined as a group of more than 2 or 3 cancer cells and can have up to 100 cells[20,21]. Aceto et al. found that CTC clusters have 23–50 times more metastatic potential than single CTCs, although they represent only 2% to 5% of all CTCs detected in a breast cancer mouse model [20]. Animal models showed that CTC clusters arise from primary tumor vein invasion and tumor fragmentation rather than single CTC aggregation in the bloodstream [22]. It has also been found that the injection of clustered cells resulted in lower OS in mice compared to the injection of single CTCs (12.7 versus 15.7 weeks, $p < 0.016$)[20]. In the same mouse model, CTC clusters were cleared from circulation at least three times more rapidly than single CTCs (half-life: 6–10 min for clusters versus 25–30 min for single CTCs)[20].

In the clinical validation cohort, we only found CMC clusters. Indeed, CTC clusters seem to be more frequently composed of mesenchymal cells rather than epithelial ones[23]. There was a higher CTC cluster count in M1TN patients than in M0 and M1TP. Although the differences did not reach statistical significance, which was probably due to the small sample size, they seem clinically relevant. In the M1TP group, we found no clusters, which may point to the efficacy of the systemic treatment.

6.6. CTC CORRELATION WITH STAGING

The correlation between CTC presence and CTC counts to staging, particularly to N1 and M1 status, has been amply demonstrated[8,10,19,24–29]. Mesenchymal CTCs expressing vimentin also correlate with a more advanced stage[8].

As previously mentioned, in the validation patient cohort we found no significant difference in CC detection rates, but we found a correlation between M status and CC counts. M1TN patients had 31.8 times more total CCs and 488 times more CMCs than M1TP patients, and 15.9 times more CCs and 31 times more CMCs than M0 patients. However, we found no differences in CTC counts according to patient characteristics like N stage, T stage, tumor histological subtypes, smoking, obesity, hypertension, or diabetes.

6.7. CTC CORRELATION WITH OUTCOMES

The correlation between CTCs and survival outcomes remains uncertain. In the systematic review, we found six papers addressing this subject, with conflicting results. All had short follow-ups and the survival outcomes were not the primary endpoints. Although CTC detection was significantly associated with decreased OS by Bluemke et al.[24], and PFS by Nel et al.[30], there were two studies that did not arrive at the same conclusion[25,31]. CTC counts were inversely correlated with OS by Basso et al.[32].

In our RUBYchip™ validation study, we found that patients with 5 or more CCs, 5 or more CMCs, and who presented CMC clusters had a significantly lower OS. The impact of CTC presence and counts on RCC survival was also proven in other studies[15,24,32–36]. In one other study, patients with > 0.12 CTCs/mL annually, had shorter overall survival (median 17.0 v 21.1 months, $p<0.001$)[34]. In yet another study, patients with mesenchymal CTCs had slightly decreased survival (HR 1.2 (1.1-1.4, $p=0.005$)[37]. In a recent study by Wang et al., postoperative total CTC counts higher than 6, the presence of post-operative mesenchymal CTCs, and the post-operative presence of CTC-WBC clusters significantly correlated with recurrence and metastasis rates[35]. In a study in M1 RCC, patients with total CTC counts of >3 had shorter OS, with a median of 13.8 versus 52.8 months on multivariate analysis (HR 1.67, 95%CI 0.95-2.93, $p=0.003$)[33]. Given the wide heterogeneity of detection and study methods, there is still no standard cut-off for CTC counts for prognostic purposes.

The same correlation with survival outcomes was found in other cancers. In colorectal cancer, more than 3 CTCs/7.5 mL was associated with reduced survival[38,39]. CTC-positive pancreatic and esophageal cancer patients were also shown to have a higher risk of tumor progression and death[40,41]. OS was also correlated to higher CTC counts in a meta-analysis of gastric cancer studies[40].

Further studies designed to study the prognostic impact of CTCs as primary endpoints are necessary before this biomarker can become a part of clinical decision pathways.

6.8. CTC CORRELATION WITH SURGICAL MANIPULATION AND POSTOPERATIVE KINETICS

An association between surgical manipulation in cancer surgery and CTC release has been demonstrated in some cancer types[42–44].

In our systematic review, four studies were found reporting a CTC increase after RCC surgery[24,45–47] and only one showed no variation in cell counts[27].

Two studies found a post-op CTC detection rate rise on D1, followed by a decrease after one week, but still above the pre-op rates[45,46]. Two later studies revealed CTCs in every patient with localized disease, both before and after surgery[9,10]. These studies with 100% detection rates suggest that with a sufficiently sensitive detection method, all cancer patients present CTCs. Thus, CTC counts are the true biomarker to consider for eventual future clinical use.

In our RCT we found no increase in CC release with the surgical manipulation of LRN. This is in line with the findings by Haga et al., where no difference in CTC counts was encountered after LRN, unlike in open RN (7.7 ± 6.8 to 22.5 ± 26.3,$p<0.05$)[10]. Therefore, the findings by both Haga et al. and our RCT suggest that minimally invasive surgery reduces CC release. Haga et al. were the only ones to compare CTC release among different surgical approaches. Only open RN showed increased post-op CTC counts on multivariable analysis, unlike

laparoscopic PN, open PN, and LRN, although the first group had a very significant selection bias towards increased TNM. Properly designed prospective comparative studies are needed to answer the question of whether the surgical approach can reduce CTC release.

Two previous studies reported an increase in CTC detection rates after surgical manipulation in kidney cancer surgery, but they did not show results for CTC counts[45,46].

The RCT also showed a progressive reduction in CMC counts over time. This reduction was only significant in D1 and in the C group. This was probably due to a random higher baseline count in the C group compared to the NT group and a small sample size. These findings are concordant with the work of Wang et al., the only to have also studied post-op CTC count kinetics, who found a progressive CTC count decrease during follow-up in patients with localized RCC[9]. They also found that patients who remained metastasis-free showed a progressive decrease in CTC counts over time, unlike patients who developed metastases, which showed an increase in CTC counts[9]. On the RCT, we also found a progressive CTC reduction over time, but it was not significant, probably due to low initial counts and small sample size.

Most studies have shown a high proportion of patients with CC persistence after radical surgery, some remaining relapse-free during follow-up. These findings remain to be explained and further studied.

6.9. IMPACT OF NO-TOUCH RN ON CTC RELEASE

Our RCT showed that a no-touch LRN does not reduce CTCs and CMCs release nor impact survival. A significant difference in CMC counts in S1 was found in the NT group, but absolute and relative variations in CTC counts were not different between the groups. This finding is in line with two RCTs in colon cancer surgery, which did not demonstrate a significant difference in the outcomes of a no-touch colon resection[48,49]. Conversely, a study on cervical cancer showed better 5-year

DFS (adjusted hazard ratio [HR], 0.202; 95% CI, 0.069-0.594; p=0.004) and 5-year OS (adjusted HR, 0.163; 95% CI, 0.035-0.748; p=0.020) for a no-touch laparoscopic radical hysterectomy, compared with a conventional technique[50]. In lung cancer, a prospective trial also showed a reduction in CTC detection rates after a no-touch pulmonary wedge resection compared to a conventional one(12.5 vs. 85.7%, p=0.02, respectively)[51].

The effect of surgery on CTC release and kinetics is still poorly understood and of uncertain clinical significance. Nonetheless, our RCT proves no advantage in a no-touch technique with early pedicle ligation. Does this mean that the last of the Robson principles has fallen? More adequately sized studies are warranted to definitively answer this question.

The RCT did show that the NT technique for LRN was faster compared to the C technique, with no difference in safety profile. NT RN was 23 minutes faster on average than the C technique. This is an advantage in itself that can favor the choice for this technique.

6.10. CTC COUNT CORRELATIONS WITH CLINICAL VARIABLES

In the RUBYchip™ validation study, the correlation of CC counts with different clinical variables was accessed. A very strong positive correlation was found between INR and all CC counts, especially in metastatic patients. This may be related to the known prothrombotic state in cancer patients, which may cause coagulation factor consumption[41]. The EMT process can also cause overexpression of tissue-factor in CTCs, conferring procoagulant activity which may contribute to metastasis[41,52]. Contrary to these findings, no significant association between activated partial thromboplastin clotting time (aPTT) or prothrombin time (PT) and CTC counts was encountered by Dirix et al.[53].

We also found that patient weight and BMI showed strong positive correlations with CMC counts in the M0 group. Age showed a moderate positive correlation,

consistent with all CC counts. We can hypothesize that age may hinder immunity, which, associated with general patient frailty, can promote CTC survival.

CTCs are known to interact with other blood cells, so we looked for such relationships. We found that leukocyte counts are moderately and positively correlated with epithelial CTCs in M1 patients, but inversely in M0 patients. This inversion was only observed for epithelial CTCs and not for CMCs or clusters. Tumor-associated neutrophils (TAN) have been shown to contribute to CTC survival by suppressing peripheral leukocyte activation in advanced cancer[54]. Furthermore, CTCs seem to develop impaired interactions with T lymphocytes and natural killer (NK) cells, which protect them from the recognition and cytotoxic activity of the immune system[55]. CTC conjugation with platelets, leukocytes, neutrophils, and tumor-associated macrophages (TAM) to form heterotypic CTC clusters confers a greater metastatic potential[55,56].

The neutrophil-to-lymphocyte ratio (NLR) correlated positively with total CCs, CTCs, CMCs, and CMC clusters in our cohort, particularly in the metastatic group. In our M1 group, increased neutrophil counts also correlated strongly with epithelial CTCs. In a previous study by Peyton et al, elevation in absolute neutrophil count, and NLR >4 were independent predictors of decreased survival in RCC ($p<0.05$)[57]. CRP levels also positively correlated with epithelial CTC counts. These findings appear to show that an elevation in inflammatory parameters correlates with increased CTC counts. In ovarian cancer, CTC-positive patients showed higher CRP compared to CTC-negative patients, with a median of 4.33 (IQR 1.46-7.51) vs. 1.52 (IQR 0.50-4.50), $p=0.001$[58]. In other cancer types, CRP was shown to correlate to survival outcomes and response to chemotherapy[59].

Serum platelet counts were inversely correlated to total CCs, CTCs, and CMC cluster counts, but only in metastatic patients. This platelet recruitment and activation could hypothetically lead to platelet consumption, lowering platelet counts. Activated platelets may shield CTCs, protecting them from immune attack and blood flow shear forces[60–62]. Platelets coating CTCs produce MHC-I-positive vesicles which may help CTCs escape recognition by NK and T cells[63]. A similar

finding was reported in a 2022 paper by Dirix et al. in advanced breast cancer, where a negative correlation between platelet count and CTC counts was found($p<0.0009$, R^2 0.167)[53]. Conversely, Guan et al. found a positive correlation between CMCs and platelet levels in RCC patients[36]. It has also been suggested that CTC-platelet interaction may also lead to EMT through TGF-ß release[64]. Hence, platelets appear to promote CTC survival and metastasis. Further studies are needed to clarify this CTC-platelet interplay.

Hemoglobin levels moderately and inversely correlated with total CC counts in the M0 group. A similar finding was reported in a prostate cancer study[65], as well as in other studies[66,67]. Serum albumin also showed a moderate and inverse correlation in M0 patients, but only with epithelial CTC counts.

Curiously, in the RCT no clinicopathological or laboratory parameter correlated to CC counts. Imagiological parameters like tumor diameter, tumor volume, tumor parenchymal contact area, or tumor attenuation value did not show a correlation to CC counts either.

6.11. ARE CMCS REALLY CTCS? — THE RISE OF AN INTRIGUING QUESTION

The scientific community has been considering DAPI+/CD45-/CK-/Vim+ cells to be mesenchymal CTCs. This was the definition we used in the validation study. However, some controversy exists in the definition of mesenchymal CTCs. Vimentin is the most commonly used marker for mesenchymal cell identification, but other markers like N-cadherin, O-cadherin, Fibronectin, Serpin peptidase inhibitor, and Twist have been chosen[21]. No marker or panel of markers has yet been standardized to definitively identify EMT or mesenchymal CTCs.

In the RCT, the healthy control group showed no CCs as expected. However, the chronic inflammation control group revealed a significant number of CMCs in all patients, despite zero CTC counts. Oncocytoma patients also had no CTCs but showed elevated numbers of CMCs. Furthermore, there was no difference

between CMC counts in this group and in both RCC and oncocytoma patients. This was a very surprising finding since control groups in the previous literature showed no CTC or CMC presence with the criteria used in the present study[15]. For this reason, we opted to call these cells CMCs and not mesenchymal CTCs. To our knowledge, no study to date has, in fact, included a chronic inflammation control group. Of note, none of these patients developed cancer during the follow-up period, one that could have been occult at the time of sample collection.

These interesting findings raise the important question of the origin and nature of these CMCs. Are they indeed CTCs or are they, for instance, a subset of WBC without CD45 or CAFs?

If they are WBCs, these findings may pave the way to important findings on the already-known interplay between cancer and inflammation[36]. The association between CMC counts and MAP scores found in the clinical trial further points in this direction. In the modern era where immunotherapy, with immune checkpoint inhibitors, is the first-line approach to the systemic therapy of advanced RCC, characterizing and monitoring these CMC counts may prove to be a valuable biomarker for treatment selection and monitoring.

It has also been suggested that some CMCs could in fact be circulating cancer-associated fibroblasts (cCAF)[68]. Curiously, we found spindle-shaped vimentin-positive cells in some samples of the validation study, which we considered as eventual cCAF and did not count as CTCs. We only considered CTCs cells positive for vimentin and with specific cytomorphological features like round/ovoid shape, big nuclei, and high nucleus-to-cytoplasm ratio.

This cell population of CMCs may even comprise a mix of all the mentioned cell types. Indeed, the characterization of CCs with only four markers is a limitation of most CTC detection techniques[15].

In any case, one relevant finding in the RCT is that irrespective of CMCs' nature, their levels were significantly reduced after radical surgery. Whichever specific cell type and function, they seem to originate from the tumor and surgery seems to be

effective in reducing them, although the clinical significance of this finding is uncertain.

For all these reasons, caution should be used in classifying CMCs as CTCs. Future CMC downstream analysis studies and improvements in marker selection are warranted to elucidate the true nature of these cells.

It is important to remember that many platforms used in early RCC CTC research did not properly detect mesenchymal cells. Hence, CC isolation platforms capable of detecting CMCs, as well as CMC clusters, like the one used in this article, should be used in future RCC research. Further characterization of these CC subpopulations, for instance with downstream analysis using DNA and RNA sequencing, may improve our understanding of their nature and clinical relevance[23]. Furthermore, a more comprehensive analysis of CCs and their interaction with the immune system can improve our understanding of RCC and improve future treatment protocols, particularly in the current era of immune checkpoint inhibitors as the main systemic therapy agents for advanced RCC.

6.12. PROJECT LIMITATIONS

This project had several limitations. The RUCYchip™ validation study had a small sample size, making it underpowered for most analyses. Despite the very limited clinical conclusions that can be drawn from such a small patient cohort, this study found a positive correlation between CTC counts and both staging and prognosis.

The RCT's main limitations were the small sample size and the concomitant low power to detect small differences in CTC counts between groups and time points. Additionally, CC downstream analysis would have been useful to better characterize CC subpopulations, and a longer follow-up time is waited for better survival analysis.

6.13. CONCLUSIONS

This research project arrived at the following conclusions:

- CTC research in RCC is still in an exploratory phase and no standard detection technique or molecular marker has been found. Further research should focus on improving CTC detection accuracy, standardization of definitions and detection methods, and comprehensive downstream analysis of captured cells.
- The RUBYchip™ microfluidic size-based CTC detection device was successfully validated for RCC and proved to be an effective and reproducible way to isolate CTCs from RCC patients. It had high detection rates with short processing times. The device identifies the different CTC phenotypes, namely CMCs and CMC clusters.
- M1TN patients had significantly more CMCs than M0 or M1TP patients. M1TP patients did not have a significant difference in CC counts compared to M0 patients. Patients with ≥5 CTCs and ≥5 CMCs have worse OS.
- NT LRN did not reduce CC release or improve survival compared to the C technique. However, NT LRN proved to be faster and as safe as C LRN. Minimally invasive surgery seems to reduce CC release in RCC.
- CMCs were found in chronic inflammation and oncocytoma patients and decreased after surgery, suggesting tumor origin but not CTC status. Caution should be used in classifying CMCs as CTCs. Future CMC downstream analysis studies and improvements in marker selection are warranted to elucidate the true nature of these cells.

6.14. FUTURE PERSPECTIVES

A clinically useful RCC biomarker remains elusive. The future of liquid biopsies in cancer is promising and it is widely believed that liquid biopsies will play a crucial role in cancer diagnosis and in treatment decisions and monitoring in the coming years.

CTCs may prove to be the missing RCC biomarker, but so far the lack of robust evidence and technical standardization restricts its clinical application. Despite all the research and technological development in the field, the detection and molecular characterization of CTCs remains technically challenging.

Further investigation is needed to identify effective molecular markers and develop highly sensitive, reliable, standardized, and cost-effective techniques for detecting CTCs in RCC so that they can be clinically used as diagnostic, prognostic, and treatment management tools.

The CITO-CERENE Project is set to continue its course. The next step will be to answer the question of the nature of the CMCs encountered in the noncancer control groups. The objective will be to do the downstream analysis of the captured CTCs and CMCs to better understand their biology and clinical relevance. Experiments with FISH probes for VHL deletion and mutations, as well as immunofluorescence experiments with a set of markers to explore WBC and platelet markers, are being designed.

Liquid biopsy biomarker research is an exciting field, holding the promise of important breakthroughs in the near future.

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7. RESEARCH ARTICLE 1 — PRINTED VERSION: Circulating tumor cell detection methods in renal cell carcinoma: A systematic review

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Circulating tumor cell detection methods in renal cell carcinoma: A systematic review

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ABSTRACT

Circulating tumor cells (CTCs) have a potential role as the missing renal cell carcinoma (RCC) biomarker. However, the available evidence is limited, and detection methods lack standardization, hindering clinical use. We performed a systematic review on CTC enrichment and detection methods, and its role as a biomarker in RCC. Full-text screening identified 54 studies. Reviewed studies showed wide heterogeneity, low evidence level, and high risk of bias. Various CTC detection platforms and molecular markers have been used, but none has proven to be superior. CTC detection and CTC count seem to correlate with staging and survival outcomes, although evidence is inconsistent. CTC research is still in an exploratory phase, particularly in RCC. Further studies are still necessary to achieve a standardization of techniques, molecular markers, CTC definitions, and terminology. This is essential to ascertain the role of CTCs as a biomarker and guide future liquid biopsy research in RCC.

1. Introduction

The presence of metastases is the most important prognostic factor in cancer patients. (Sun et al., 2011; Gorges and Pantel, 2013) One-third of patients with renal cell carcinoma (RCC) present with regional or metastatic disease and up to 40 % of those treated for localized disease ultimately develop metastases or, less frequently, local recurrence.(Lin et al., 2019; Santoni et al., 2019; Wiechno et al., 2018) RCC diagnosis and follow-up are still based on the identification of tumor masses on cross-sectional imaging. The threshold for identifying a renal mass or lymph node metastasis is approximately 1 cm, which implies that we can only detect the disease when more than 10^8 cancer cells have developed. (Monte and Del Monte, 2009) There is currently no biomarker capable of detecting early localized disease or early relapses before gross masses are visible on imaging.(Li et al., 2005) To improve RCC patients' survival, it is paramount to identify a biomarker able to accurately diagnose both early localized and micrometastatic disease.

Liquid biopsies, namely circulating tumor cells (CTCs), are an emerging non-invasive tool for diagnosing and monitoring disease status in several urogenital cancers.(Cimadamore et al., 2019) In 1869, Ashworth was the first to report the presence of cells similar to the primary tumor in *post-mortem* blood samples.(Ashworth, 1869) CTCs are tumor cells that have migrated from a primary tumor or metastatic site through passive shedding or by the dynamic process of stromal invasion and resultant intravasation into the bloodstream.(Gorin et al., 2017; Wang et al., 2019) Only about 0.01 % of CTCs are thought to form metastases at remote sites.(Blumke et al., 2005)

The potential clinical value of CTCs has been explored in several tumor types.(Liberko et al., 2013) It has been shown that CTC count (CTCn) is an independent prognostic marker for metastatic breast cancer and a predictor of recurrence and disease progression.(Cristofanilli et al., 2004; Zhang et al., 2017) In metastatic castration-resistant prostate cancer and metastatic colorectal cancer, an association has also been shown between CTCn and overall survival (OS), cancer-specific survival (CSS), and progression-free survival (PFS).(de Bono et al., 2008; Cohen et al., 2008)

CTC analysis typically includes three steps: 1) blood sample preparation and CTC enrichment; 2) CTC isolation and detection; and 3)

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further CTC characterization, for instance through gene expression profiling.(Small et al., 2012)

There is currently no standard detection method for CTCs. These are extremely rare compared to whole blood cells, with an estimated CTCn of one CTC per billion normal blood cells in metastatic cancer patients. (Maheswaran and Haber, 2010) Additionally, a proportion of these cells is lost during sample processing, making their detection difficult and utterly reliant on the sensitivity and specificity of the methods used. (Bankó et al., 2019) Furthermore, the lack of specific RCC molecular markers has hindered CTC research in this tumor compared to other epithelial tumors.(Liu et al., 2016) Previously reported CTC detection rate (DR) is relatively poor and there is an ongoing search for a method that is highly sensitive and specific, while at the same time reproducible, inexpensive, and simple to use.

Cellsearch® is the only FDA-approved CTC detection platform. However, it is deemed inappropriate for RCC due to the lack of epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) expression in many CTCs which have undergone epithelial to mesenchymal transition (EMT).(Liu et al., 2016) Data suggests that many RCC cells undergo early EMT, with the development of resistance to anoikis and other apoptotic signals.(Small et al., 2012; Montironi et al., 2015) This feature is illustrated by a gain in expression of mesenchymal markers, such as vimentin.(Broncy and Paterlini-Bréchot, 2018)

The great heterogeneity of techniques and results calls for a systematic review of the literature to structure current findings and possibly guide future research to improve knowledge of CTCs in RCC and their clinical applicability. Herein are described CTC enrichment and detection methods used so far, their DR and CTCn, and available evidence on their value in diagnosis, staging, prognosis, and treatment response.

2. Evidence acquisition

This systematic review of published literature was performed following the Preferred Reporting Items for Systematic Review and Meta-analysis (PRISMA) guidelines for systematic reviews.(Moher et al., 2015) Methods and inclusion criteria were stated in advance and documented in a protocol registered in the International Prospective Register of Systematic Reviews (PROSPERO) International Registry — CRD42017074226.

A literature search was conducted on Embase®, Web of Science®, Ovid MEDLINE®, Ovid MEDLINE® Daily and Ovid MEDLINE® Epub Ahead of Print, and In-Process & Other Non-Indexed Citations, from inception until April 1, 2020. The complete search string is attached as supplementary material.

Literature search aimed to include published peer-reviewed articles, conference proceedings, and other grey literature. Inclusion criteria comprised studies describing CTC enrichment and detection methods from peripheral or central blood samples of RCC patients of any histology and staging. Publications in human adult patients (>18 years) with a sample size larger than one patient were considered. Exclusion criteria were as follows: review articles, editorial comments, letters to the editor, study protocols were considered wrong study design; repeated references; studies focusing on spiking experiments with cell cultures of RCC cell lines were excluded because no patients were studied; studies not reporting CTC detection rate or CTC counts were excluded as wrong outcomes; studies which did not include RCC patients (studies including only other tumor types and locations) were excluded based on wrong target population; studies focused on biomarkers other than CTCs, such as circulating endothelial cells or circulating nucleic acids, were excluded due to wrong intervention; studies with samples obtained ex-vivo or in tumor specimens were excluded due to wrong setting.

Title and abstract screening followed by full-text screening were independently conducted by three authors. Duplicates were excluded. Disagreement between authors was resolved by a fourth author. Data extraction was performed by four authors. The following data items were retrieved: study reference, study design, sample size, participant characteristics, enrichment techniques, and detection methods. Data on CTC performance as a diagnostic (CTC DR and CTCn), prognostic (OS, CSS, and PFS), and treatment response biomarker (post-op CTC DR and CTCn) according to the BEST (Biomarkers, EndpointS, and other Tools) approach was also collected. FDA-NIH Biomarker Working Group (2016)

Due to the exploratory nature and heterogeneity of selected studies, two different critical appraisal tools to assess the risk of bias were applied according to study design. For observational descriptive studies, the Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Case Series was used.(Institute et al., 2014) For observational analytical (cohort and case-control) studies, the Newcastle-Ottawa Quality Assessment Scale was applied.(Wells et al., 2015) (Appendix — tables 4.1 and 4.2)

Given the great heterogeneity in detection methods, DRs, populations, and outcomes between studies, a quantitative pooling of study results was not appropriate, and hence a qualitative analysis was performed.

3. Evidence synthesis

The literature search identified 2529 studies, 54 of which were included after screening and eligibility assessment (Fig. 1).

Retrieved evidence was organized in three topics: CTC enrichment techniques, CTC detection methods, and CTC performance as a diagnostic, prognostic, and treatment response biomarker (Fig. 2).

3.1. CTC enrichment methods

Enrichment refers to the process of concentrating these very rare cells in a sample for subsequent detection and characterization. This can be done in two ways: by positive enrichment, in which CTCs are isolated or selected from the sample, or by negative enrichment, in which the sample is depleted of blood cells. A multitude of enrichment methods have been used to date, which can be organized in four different categories: antibody- (27 studies), density- (14 studies), size- (11 studies), and electrophoresis- (1 study) based methods (Table 1 and Fig. 2).

3.1.1. Antibody-based methods

Antibody-based enrichment methods usually rely on immunomagnetic selection. For negative enrichment, CD45 is the marker typically applied for leukocyte depletion. A commonly used technique is Magnetic Cell Separation System (MACS), which couples ferromagnetic nanoparticles (magnetic beads) to CD45 antibodies.(Blumke et al., 2005; Bilkenroth et al., 2001; Meye et al., 2002; Bluemke et al., 2009; Nel et al., 2016)

The most commonly used markers for positive enrichment are EpCAM and CKs due to the epithelial origin of RCC.(Maetzel et al., 2009) CellSearch® uses ferrofluid nanoparticles coated with anti-EpCAM antibodies to retain CTCs passing through a magnetic field.(Allard et al., 2004; Lomo et al., 2006; Seideman et al., 2009; Gradilone et al., 2011; Rossi et al., 2012; Yip et al., 2014; Novikova et al., 2015; Basso et al., 2017; Bai et al., 2018)

One author used a microfluidic antibody-based device consisting of chips with geometrically arranged micro spots that promote cell-antibody binding. This device showed higher CTC capture efficiency in RCC patients with a modified NanoVelcro platform using CA9 and CD147-capture antibodies.(Liu et al., 2016)

3.1.2. Density-based methods

This technique is based on the fact that tumor cells and leukocytes have different densities, enabling their separation by centrifugation. This method was mostly used in initial studies and enables a marker-independent enrichment, yet leading to tumor cell loss and false

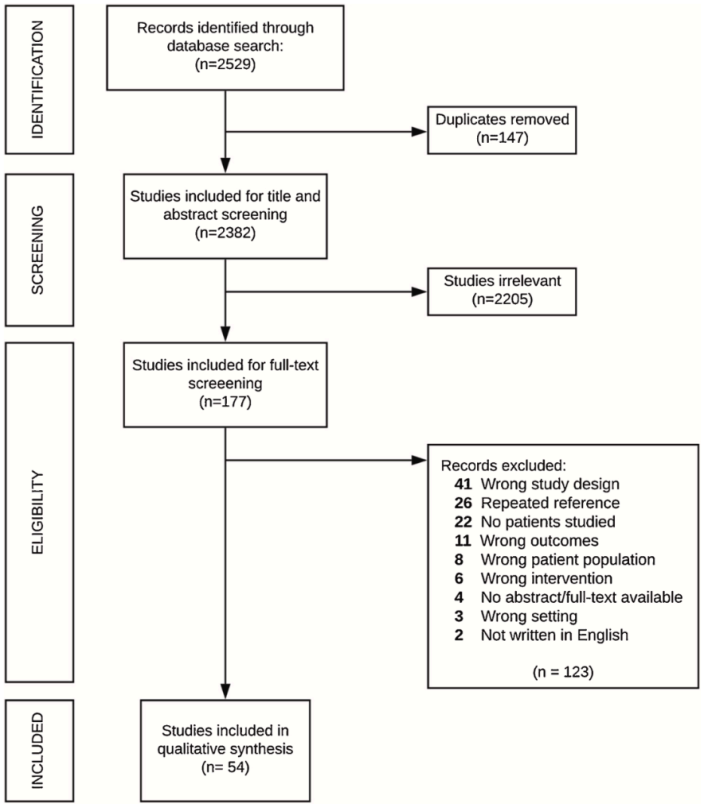

Fig. 1. Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flow diagram.

negatives.(Li et al., 2005; Glaves et al., 1988; Hioki and Sugimura, 1999; Uemura et al., 1999; McKiernan et al., 1999; Ashida et al., 2000; de la Taille et al., 2000; Uemura et al., 2003; Shimazui et al., 2003, 2004; Gilbert et al., 2006; Ramirez et al., 2012; Amato et al., 2015; Liu et al., 2017)

3.1.3. Size-based methods

This label-free cell selection method is based on the bigger size and less deformability of CTCs compared to blood cells. It has the advantage of minimizing cell loss. It also allows the sorting of viable cells for further downstream analysis. The main limitations are the risk of filter/channel clogging due to cell agglutination and losing CTCs smaller than pore sizes. Size-based enrichment can be accomplished through membrane-based(Wang et al., 2019; Bai et al., 2018; Mittal et al., 2012, 2014; El-Heliebi et al., 2013; Williams et al., 2012; Broncy et al., 2018; Gutschi et al., 2010) or microfluidic devices (Kang et al., 2018; Kim et al., 2019; Naoe et al., 2019), most commonly calibrated to capture cells bigger than 8 μm.

3.1.4. Electrophoresis-based methods

Only one article reported the use of an electrophoresis-based microfluidic positive enrichment method, the ApoStream® Rare Cell Enrichment Platform. It relies on the fact that CTCs have specific polarization charges due to different diameters, membrane area, density, conductivity, and volume. Hence, CTCs are attracted to the bottom of the device in an electrical field, while the sample flows (Gorin et al., 2015).

3.2. CTC detection methods

After sample enrichment, CTC detection is performed by one of five different techniques: immunocytochemistry (32 articles), reverse transcriptase-polymerase chain reaction (RT-PCR) (13 articles), cytomorphological criteria (11 articles), flow cytometry immunofluorescence (5 articles), and fluorescence in situ hybridization (FISH) (4 articles) (Fig. 2).

3.2.1. Immunocytochemical analysis — chromogenic or immunofluorescence

Most research groups use immunocytochemistry for CTC detection, which can be performed in one of two ways. The first is chromogenic immunocytochemistry, which uses enzyme-coupled antibodies to catalyze subtracts into an insoluble and chromogenic precipitate visible on standard light microscopy.(Blumke et al., 2005; Bilkenroth et al., 2001; Meye et al., 2002; Bluemke et al., 2009; El-Heliebi et al., 2013; Gutschi et al., 2010; Theil et al., 2014) The second is immunofluorescence, which uses antibodies coupled to a fluorophore for visualization on fluorescence microscopy.(Liu et al., 2016; Nel et al., 2016; Allard et al., 2004; Lomo et al., 2006; Seideman et al., 2009; Gradilone et al., 2011; Rossi et al., 2012; Yip et al., 2014; Novikova et al., 2015; Basso et al., 2017; Bai et al., 2018; Glaves et al., 1988; Ramirez et al., 2012; Mittal et al., 2012, 2014; Kim et al., 2019; Fehm et al., 2002; Zhang et al., 2016; Arafat et al., 2017; Xing et al., 2018; Emamekhoo et al., 2020) Molecular markers used vary widely, being CKs the most common (Table 1).

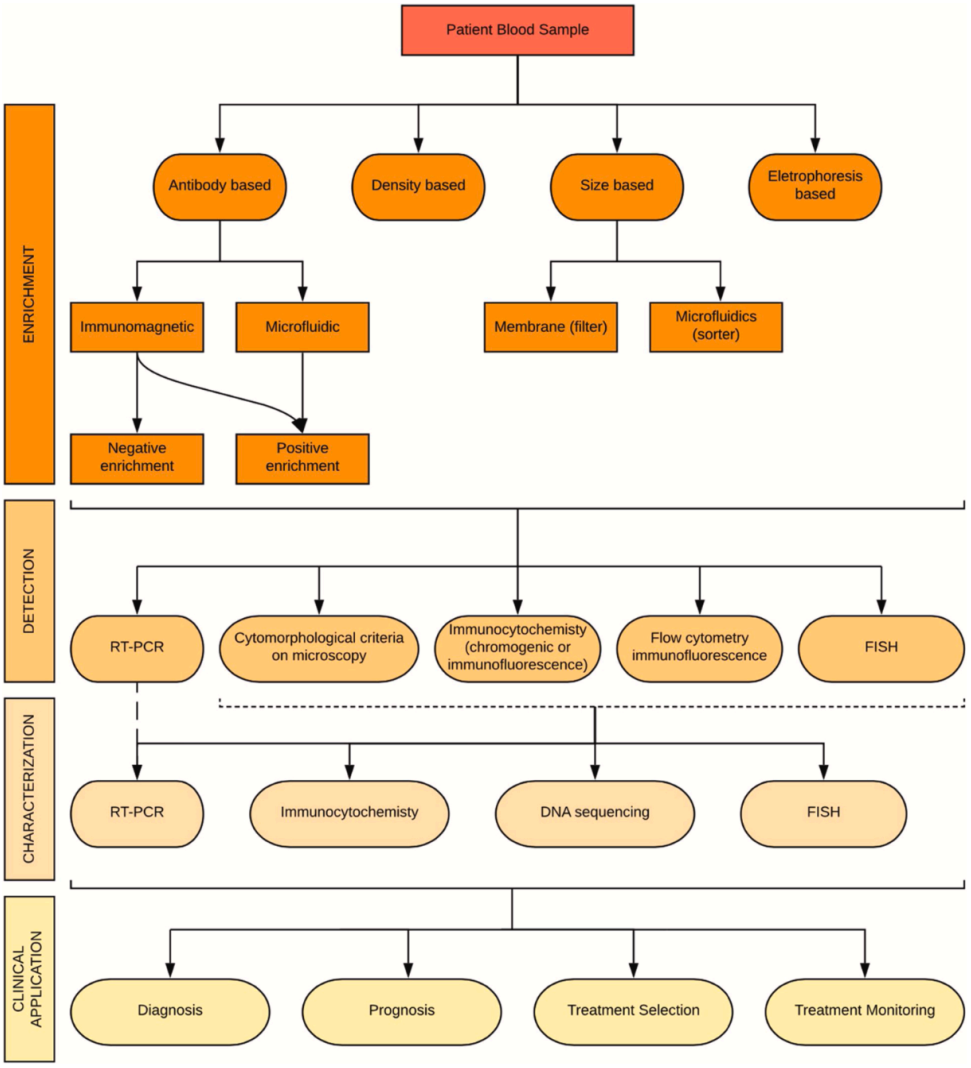

Fig. 2. CTCs enrichment, detection and characterization methods flow chart and their potential role as a biomarker.

3.2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Most initial works used RT-PCR for CTC detection. This technique does not detect actual CTCs, but rather infers their presence by detecting circulating RNA of specific RCC markers, such as CK19(Hioki and Sugimura, 1999), carbonic anhydrase (CA) 9(Uemura et al., 1999; McKiernan et al., 1999; de la Taille et al., 2000; Uemura et al., 2003; Gilbert et al., 2006; Ohlmann et al., 2006), Von Hippel Lindau (VHL) gene mutation(Ashida et al., 2000), prostate-specific membrane antigen (PSMA)(de la Taille et al., 2000), cadherin 6(Li et al., 2005; Shimazui et al., 2003, 2004), CK8(Iacovelli et al., 2011), or vimentin.(Iacovelli et al., 2011)

3.2.3. Cytomorphological criteria on microscopy

Many authors rely on cytomorphological criteria for CTC identification, most often as a complementary analysis of immunocytochemistry.(Blumke et al., 2005; Liu et al., 2016; Meye et al., 2002; Bluemke et al., 2009; Bai et al., 2018; Liu et al., 2017; El-Heliebi et al., 2013; Broncy et al., 2018; Kang et al., 2018; Kim et al., 2019) Although not unanimous, the usual criteria for CTC definition are 1) large cell size, 2) large nuclear size with high nuclear/cytoplasmic ratio, 3) irregular nuclear membrane, and 4) cell irregularity.

3.2.4. Flow cytometry immunofluorescence

Flow cytometry immunofluorescence has recently been reported as a technique allowing faster, cheaper, and more automated sample processing.(Naoe et al., 2019; Haga et al., 2020; Ionescu-Zanetti et al., 2015; Tseng et al., 2015; Desotelle et al., 2016)

3.2.5. Fluorescence in situ hybridization (FISH)

FISH probes targeting the VHL gene and chromosome 8 aneuploidy have been used by some groups, (Gorin et al., 2015; Ye et al., 2019; Wu et al., 2019) with one group using RNA FISH for several markers.(Wang et al., 2019)

Table 1
Studies assessing CTCs in RCC patients - CTC detection rates and counts.

Reference (author/ year)	Enrichment / Isolation Method	Detection Method	Molecular Markers	Patients (n)	Staging (% M1)	CTC DR (%)	CTCn (median, [range], units)
Glaves et al., 1988	Density based - DGC	Immunocytochemical (IF)	CK	10	30	80.0	160.5 [0–7309]/mL*
Hioki and Sugimura, 1999	Density based - DGC	RT-PCR	CK19	19	21	47	–
Uemura et al., 1999	Density based - DGC	RT-PCR	CA9	42	NA	76.2	–
McKiernan et al., 1999	Density based - DGC	RT-PCR	CA9	42	21.4	49.0	–
Ashida et al., 2000	Density based - DGC	RT-PCR	VHL	20	15	75.0	–
de la Taille et al., 2000	Density based - DGC	RT-PCR	CA9, PSMA	59	4.0	18.6	–
Bilkenroth et al., 2001	Antibody based - IM(-)	Immunocytochemical (CG)	CK	59	13.6	32.2	8.0 [1–38]/8 mL
Meye et al., 2002	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK	24	16.7	41.7	5.0 [1–13]/16 mL*
Fehm et al., 2002	Antibody based - IM(-)	Immunocytochemical (IF)	CK	4	0	100.0	–
Uemura et al., 2003	Density based - DGC	RT-PCR	CA9	38	31.6	18.5 OA 52.9	–
Shimazui et al., 2003	Density based - DGC	RT-PCR	Cadherin 6	87	13.8	M0 45.0 M1 70.4 OA 47.0	–
Shimazui et al., 2004	Density based - DGC	RT-PCR	Cadherin 6	66	34.8	M0 34.9 M1 69.9	–
Allard et al., 2004	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	11	100	–	1 ± 1/7.5 mL
Blumke et al., 2005	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK or CK8/18	214	NA	37.4	0.56 [0.06–4.75]/mL
Li et al., 2005	Density based - DGC	RT-PCR	Cadherin 6	46	23.9	45.7	–
Ohlmann et al., 2006	Not done	RT-PCR	CA9	24	0	45.8	–
Lomo et al., 2006†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	10	NA	–	2.6(±1.4) [1–5]/7.5 mL
Gilbert et al., 2006	Density based - DGC	RT-PCR	CA9	36	0	33.3	–
Bluemke et al., 2009	Antibody based - IM(-)	Immunocytochemical (CG) Cytomorphological analysis	CK or CK8/18	154	NA	37.1	6.0 [1–51]/16 mL
Seideman et al., 2009†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	12	NA	92.0	–
Gutschi et al., 2010†	Size based - MB	Immunocytochemical (CG)	–	19	21	63.2	–
Gradilone et al., 2011	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19 CD44	25	100	16.0	1.0 [1–4]/2 mL
Iacovelli et al., 2011†	Antibody based - IM	RT-PCR	CK8, Vimentin	18	100	50.0	–
Rossi et al., 2012	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	53	13.2	58.5	2.0 [1–141]/7.5 mL
Williams et al., 2012†	Size based - MB	–	–	7	NA	100.0	[1–128]
Mittal et al., 2012†	Size based - MB	Immunocytochemical (IF)	CK	26	100	50.0	[0–58]/mL
Ramirez et al., 2012†	Density based - DGC	Immunocytochemical (IF)	EpCAM, CA9, CD10, EGFR	4	100	75.0	[1–6]/3 mL
El-Heliebi et al., 2013	Size based - MB	Cytomorphological analysis Immunocytochemical (CG)	CA9	30	3.3	29 CA9 16.2	3.0 [1–62]/8 mL
Yip et al., 2014†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	55	100.0	16.1	–
Mittal et al., 2014†	Size based - MB	Immunocytochemical (IF)	CK	17	100	53.0	OA 1/mL
Theil et al., 2014†	Antibody based - IM(+)	Immunocytochemical (CG)	EpCAM, CK, MUC1	20	NA	91.6	M0 5.3 [0–11]/7.5 mL M1 9.6 [2–44]/7.5 mL EpCAM+ 6.7 [2–15]/ 7.5 mL EpCAM+/MUC1+ 5.1 [1–8]/7.5 mL
Gorin et al., 2015†	Electrophoresis based - Microfluidic	FISH	VHL gene	30	100	26.7	–
Novikova et al., 2015†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	6	100	67.0	[2–5]/7.5 mL

(*continued on next page*)

Table 1 (*continued*)

Reference (author/ year)	Enrichment / Isolation Method	Detection Method	Molecular Markers	Patients (n)	Staging (% M1)	CTC DR (%)	CTCn (median, [range], units)
Amato et al., 2015†	Density based - DGC	Cytomorphological analysis Immunocytochemical (IF)	CK	4	100	100.0	1.6/mL 9.1/mL
Ionescu-Zanetti et al., 2015†	Antibody based - IM(+)	Flow cytometry IF	EpCAM	6	NA	100.0	248 [14–731]/15 mL*
Tseng et al., 2015†	Antibody based - IM(+)	Flow cytometry IF	EpCAM	8	100	100.0	260 [65–1413]/15 mL *
Liu et al., 2016	Antibody based - IM(+)	Immunocytochemical (IF) Cytomorphological analysis	CK, CA9, CD147, vimentin	76	15.8	94.7	12.8 (±6.9)/2 mL
Nel et al., 2016	Antibody based - IM(-)	Immunocytochemical (IF)	CK,n-cadherin, CD133	14	100.0	64.3	7.2/1000 PBMNC
Zhang et al., 2016	Antibody based - IIM(+)	Immunocytochemical (IF)	c-MET, CK	10	100	10.0	0 [0–3]/7.5 mL
Desotelle et al., 2016†	Not reported	Flow cytometry IF	EpCAM, CA9, CA12, PAX8, CK	20	NA	–	CK+/CA9+ 24.5 [0–5410]/7.5 mL CK+/EpCAM+ 5.5 [1–231]/7.5 mL
Liu et al., 2017	Density based - DGC	Cytomorphological analysis Acridine orange fluorescence	–	139	19.4	OA 13.67 M0 8.9 M1 33.3	–
Arafat et al., 2017†	Antibody based - IM(+)	Immunocytochemical (IF)	CA9,CA12, PAX8	27	100	96.2	–
Basso et al., 2017†	Antibody based - IM(+)	Immunocytochemical (IF)	CK8, CK18, CK19	195	100	46.6	2 [1–263]/7.5 mL
Broncy et al., 2018	Size based - MB	Cytomorphological analysis DNA amplification nested PCR	–	30	6.7	96.7	–
Xing et al., 2018	Antibody based - IM(+)	Immunocytochemical (IF)	CK	7	42.9	57.1	1.29 [0–3]/mL*
Bai et al., 2018	Antibody based - IM(+) Size based - MB	Immunocytochemical (IF) Cytomorphological analysis	CK8, CK18, CK19 –	36	27.8	36.1	–
Kang et al., 2018	Size based - Microfluidics	Immunocytochemical (IF) Cytomorphological analysis	EpCAM, CK, CD10	48	33.3	27.1	1 [0–5]/mL *
Ye 2018	Antibody based - IM(-)	FISH Immunocytochemical (IF)	CEP8, CK18, EpCAM	74	NA	91.9	8 [1–52]/7.5 mL
Wang et al., 2019	Size based - MB	RNA FISH	EpCAM, CK8, CK18, CK19, Vimentin, Twist, Beclin-1	69	0	100.0	Epithelial 8.21 (±4.77)/7.5 mL Mesenchymal 2.30 (±1.41)/7.5 mL Mixed 5.20(±2.24)/ 7.5 mL
Kim et al., 2019	Size based - Microfluidics	Immunocytochemical (IF) Cytomorphological analysis	EpCAM, CK	34	55.9	61.8	2.0 [1–6]/5 mL
Naoe et al., 2019	Size based - Microfluidics	Flow cytometry IF	CA9	13	38.5	69.2	2/4 mL*
Wu et al., 2019	Antibody based - IM(-)	FISH, Immunocytochemical (IF)	CEP8, CK18	8	0	75.0	5.5 [0–12]/7.5 mL
Haga et al., 2020	Antibody based - IM(-)	Flow cytometry IF	EpCAM, CK	54	8.3	100.0	LRN 3.4 ± 4.2/4 mL ORN 7.7 ± 6.8/4 mL LPN 3.4 ± 4.1/4 mL OPN 6.0 ± 7.6/4 mL
Emamekhoo et al., 2020†	Antibody based - IM(+)	Immunocytochemical (IF)	CA9, CA12, CK, CD45/34/ 66b	26	100	100	CK+ 5 [0–53/mL] CA12+ 1/mL CA12+/CK+ 7 [0–102]/mL

CEP8 = chromosome 8 centromere probe; **CG** = chromogenic; **CK** = cytokeratin; **CSS** = cancer specific survival; **CTCn** = circulating tumor cell count; **DGC** = density gradient centrifugation; **DR** = detection rate; **EpCAM** = epithelial cell adhesion molecule; **FISH**= fluorescence in-situ hybridization; **IF** = immunofluorescence; **IM (+)** = immunomagnetic positive enrichment; **IM(-)** = immunomagnetic negative enrichment; **LPN** = laparoscopic partial nephrectomy; **LRN** = laparoscopic radical nephrectomy; **MB** = membrane based; **NA** = not applicable; **OA** = overall; **OPN** = open partial nephrectomy; **ORN** = open radical nephrectomy; **OS** = overall survival; **PFS** = progression-free survival.

† Grey Literature.

* Calculated median based on data provided.

3.3. CTC performance as a biomarker

CTCs are being studied as a diagnostic (Table 1), prognostic (Table 2), and treatment response (Table 3) biomarker.

3.3.1. Diagnostic biomarker

3.3.1.1. CTC detection rate (CTC DR). CTC DR varied widely between techniques, molecular markers, and research groups, ranging from 10 to 100 %, with a median of 57 % (interquartile range [IQR] 55).

Research groups using MACS followed by immunocytochemistry reported similar DRs, ranging from 32.2–41.7%.(Blumke et al., 2005; Bilkenroth et al., 2001; Meye et al., 2002; Bluemke et al., 2009) On the other hand, studies conducted with CellSearch® showed DRs ranging from 16.0–92.0%.(Seideman et al., 2009; Gradilone et al., 2011; Rossi et al., 2012; Yip et al., 2014; Novikova et al., 2015; Basso et al., 2017; Bai et al., 2018; Iacovelli et al., 2011) In the only study comparing techniques, ISET® showed a 36.1 % and CellSearch® a 19.4 % DR, with low consistency between both (Kappa = 0.063, p = 0.673).(Bai et al., 2018) Liu S. et al. was the only group so far to target CA9 and CD147 using an antibody-based microfluidics chip, achieving a 94.7 % DR.(Liu et al., 2016) By contrast, DR using EpCAM was only 18.6 %. More recently, (Haga et al. (2020)), as well as some grey literature(Ionescu-Zanetti et al., 2015; Tseng et al., 2015), obtained 100 % CTC DR using flow cytometry immunofluorescence.

Most groups used peripheral blood samples, but renal vein blood was also studied.(Hioki and Sugimura, 1999; Uemura et al., 1999; Ashida et al., 2000; Uemura et al., 2003) Uemura et al. found a higher DR in renal vein (33.3 %) than in peripheral blood (18.5 %).(Uemura et al., 2003) Conversely, Hioki et al. reported a 32 % DR in peripheral blood compared to 25 % in the renal vein.(Hioki and Sugimura, 1999)

Several studies found a positive correlation between DR and tumor staging.(Blumke et al., 2005; Bluemke et al., 2009; Bai et al., 2018; Hioki and Sugimura, 1999; Shimazui et al., 2003; Liu et al., 2017; Kim et al., 2019) Hioki et al. reported a 0% DR for stage 1, 25 % for stage 2, 75 % for stage 3, and 100 % for stage 4 tumors (p = 0.002).(Hioki and Sugimura, 1999) Shimazui et al. showed concordant results, with 70.4 % in M1 and 45.0 % in M0 patients (p = 0.037).(Shimazui et al., 2003) Blümke et al. reported a significant correlation with N and M status, with 42 % DR for N0, 86 % for N1, 91 % for N2 (p < 0.001), 43 % for M0, and 67 % for M1 patients (p = 0.014).(Blumke et al., 2005; Bluemke et al., 2009)

Table 2
Studies assessing correlation between CTCs and prognosis.

Reference (author year)	Prognostic Outcome Measure	Prognostic Outcome and Measure Effect Size
Gilbert et al., 2006	5y DFS (CA9 + vs. -) 5y CSS (CA9 +vs. -) 5y OS (CA9 + vs. -) (median FU 4.7y)	39.5 % **vs. 88.1 %, p = 0.048** 85.7% vs. 96.0 %, p = 0.57 76.2% vs. 72.2%, p = 0.93
Bluemke et al., 2009	OS (CK + vs. CK- before surgery) OS (CK + vs. CK- after surgery) OS (CK + vs. CK- before and after surgery) (median FU 15 mo)	**RR 2.7, p = 0.049 RR 4.3, p = 0.036 RR 2.3, p = 0.048**
Nel et al., 2016	PFS (n-cadherin + CTCn <0.35 vs. 0.35/1000 PBMNC); PFS (n-cadherin + CTC vs. no CTC) (median FU not stated)	**7 vs. 15 months, HR 0.31 (CI 0.06−1.59), p = 0.04**
Basso et al., 2017	PFS (CTC + vs. CTC -) PFS (CTC> = 3 vs. CTC < 3/7.5 mL) OS (CTC> = 3 vs. CTC < 3/7.5 mL) (median FU 31.5 mo)	**8.8 vs. 16.6 mo, HR 1.41 (CI 1.02−1.9), p = 0.03 5.8 vs. 15 mo, HR 1.99 (CI 1.28−3.03), p = 0.002 13.8 vs. 52.8 mo, HR 1.99 (CI 1.17−3.2), p = 0.003**
Bai et al., 2018	OS (CTC + vs. CTC-) CellSearch OS (CTC + vs. CTC-) ISET PFS (CTC + vs. CTC-) CellSearch PFS (CTC + vs. CTC-) ISET (median FU 36 mo)	HR 1.78 (CI 0.48−6.57), p = 0.391 h 0.57 (CI 0.18−1.78), p = 0.336 h 1.43 (CI 0.4−5.15), p = 0.581 h 1.95 (CI 0.68−5.6), p = 0.214
Kim et al., 2019	2y PFS (CTC + vs. -) 2y CSS (CTC + vs. -) (median FU 19.5 mo)	81.0 % vs. 100.0 %, p = 0.704 38.5 % vs. 49.2 %, p = 0.158

Bold when statistically significant difference.
CI = confidence interval; **CK** = cytokeratin; **CSS** = cancer-specific survival; **CTC** = circulating tumor cell; **DFS** = disease-free survival; **FU** = follow up **HR** = hazard ratio; **mo** = months; **OS** = overall survival; **PBMNC** = peripheral blood mononuclear cells; **PFS** = progression-free survival; **RR** = relative risk; **y** = years.

3.3.1.2. CTC count (CTCn). Thirty-three articles reporting CTCn were identified. Some showed an association between CTCn and clinicopathological features. Theil et al. found a significantly higher CTCn in patients with metastatic disease compared to localized disease, of 9.6 vs. 5.3 CTC/7.5 mL, respectively.(Theil et al., 2014) Liu S. et al. reported a 2.2-fold increase in CTCn between late-stage (3 and 4) and early-stage (1 and 2) disease (p < 0.001). This group additionally found higher vimentin-positive mesenchymal-type cell counts with increasing stage (p < 0.001).(Liu et al., 2016) Haga et al. also showed higher preoperative CTCn in stage 4 disease compared to other stages, both on univariable (stage 1: 3.3 ± 4.1, stage 2: 4.7 ± 4.2, stage 3: 4.7 ± 2.9, stage 4: 13.0 ± 10.8, p < 0.001) and multivariable (p < 0.001) analysis, but not in postoperative counts.(Haga et al., 2020) On the other hand, several studies failed to show this correlation.(Wang et al., 2019; Seideman et al., 2009; Mittal et al., 2012; Ohlmann et al., 2006) No correlation between RENAL score, Fuhrman grade, or venous invasion and CTCn was observed, both pre and post-operatively.(Haga et al., 2020)

3.3.2. Prognostic biomarker

Table 2 summarizes six studies that correlated CTC detection with prognostic outcomes. Four studies found a correlation between either CTC DR or CTCn and prognostic outcome measures.(Bluemke et al., 2009; Nel et al., 2016; Basso et al., 2017; Gilbert et al., 2006) Conversely, two studies showed no statistically significant correlation with survival outcomes.(Bai et al., 2018; Kim et al., 2019)

3.3.3. Treatment response biomarker

Seven studies measured CTCs in the perioperative setting (Table 3). Ashida et al. were the first to show an increased CTC DR after surgical manipulation: 11.8 % before surgery, 37.5 % on D1, 18.8 % on D7, and 18.2 % on D30.(Ashida et al., 2000) Similarly, El-Heliebi et al. reported a significant increase in CTC DR in the immediate postoperative period, from 29 % pre-op to 40 % on D1 (p = 0.04), decreasing to 33 % on D8. (El-Heliebi et al., 2013) Interestingly, two studies revealed a 100 % DR, both before and after surgery for localized disease.(Wang et al., 2019; Haga et al., 2020)

Haga et al. found a higher immediate post-op CTCn after open radical nephrectomy (ORN), with an increase from 7.7 ± 6.8–22.5 ± 26.3 (p < 0.05), but not after laparoscopic radical nephrectomy (LRN), open partial nephrectomy (OPN), or laparoscopic partial nephrectomy (LPN). (Haga et al., 2020)

Wang et al. studied CTCn kinetics in patients with localized disease at 6 and 12 months post-op.(Wang et al., 2019) Patients were stratified according to metastases development on follow-up. In the M1 group, a progressive increase in CTCn was observed, from 4.42 ± 2.35–6.33 ± 3.03 at 6 months and 9.8 ± 5.03 at 12 months (p < 0.05). In the M0 group, a progressive decrease in CTCn was reported, from 5.20 ± 2.24–4.52 ± 2.30 at 6 months and 3.63 ± 1.37 at 12 months (p < 0.05).

Only two studies compared CTC detection according to surgical technique and approach. Ohlman et al. found no difference in CTC DR between transperitoneal and retroperitoneal ORN (Ohlmann et al., 2006). Haga et al. found a significant increase in postoperative CTCn only in ORN but not in LRN, OPN, or LPN (LRN 4.8 ± 3.7, ORN 22.5 ± 26.3, LPN 7.9 ± 9.1, OPN 6.4 ± 6.3, p < 0.001 on multivariable analysis). Additionally, the authors found that tumor diameter significantly

Table 3
Studies assessing CTCs in the perioperative setting.

Reference (author/ year)	Patients (n)	Pre-op CTC DR (%)	Post-op CTC DR (%)		Pre-op CTCn (median or mean, units)		Post-op CTCn (median, [range], units)
Ashida et al., 2000	20	11.8	D1 37.5 D7 18.8 D30 18.2		—		—
Shimazui et al., 2003	87	52.9	D7–21 54.2		—		—
Ohlmann et al., 2006	24	45.8	58.3		—		—
Bluemke et al., 2009	154	37.1	D5–7 45.4		6/16 mL (range 1–51)		—
El-Heliebi et al., 2013	30	29	D1 40.0 D8 33.0		3/8 mL (range 1–62)		D1 3 [1–37]/8 mL D8 3 [1–58]/8 mL
Wang et al., 2019	69	100	6–12 M 100.0	Overall:	Epithelial 8.21 (±4.77)/7.5 mL Mesenchymal 2.30 (±1.41)/ 7.5 mL Mixed 5.20 (±2.24)/7.5 mL Epithelial 0.91 (±0.57)/7.5 mL	6M (M0 on FU):	Epithelial 1.39 (±0.84)/7.5 mL Mesenchymal 2.02 (±1.58)/ 7.5 mL Mixed 4.52 (±2.30)/7.5 mL Epithelial 2.66 (±1.78)/7.5 mL
				M0 on FU:	Mesenchymal 2.30 (±1.41)/ 7.5 mL Mixed 5.20 (±2.24)/7.5 mL Epithelial 0.85 (±0.46)/7.5 mL Mesenchymal 2.42 (±1.40)/ 7.5 mL	6M (M1 on FU): 12 M (M0 on FU):	Mesenchymal 2.66 (±1.30)/ 7.5 mL Mixed 6.33 (±3.03)/7.5 mL Epithelial 0.72 (±0.33)/7.5 mL Mesenchymal 2.47 (±1.98)/ 7.5 mL
				M1 on FU:	Mixed 4.42 (±2.35)/7.5 mL	12 M (M1 on FU):	**Mixed 3.63 (±1.37)/7.5 mL** Epithelial 2.20 (±1.83)/7.5 mL Mesenchymal 4.60 (±2.39)/ 7.5 mL **Mixed 9.80 (±5.03)/7.5 mL**
Haga et al., 2020	54	100	D0 100.0		LRN 3.4 ± 4.2/4 mL ORN 7.7 ± 6.8/4 mL LPN 3.4 ± 4.1/4 mL OPN 6.0 ± 7.6/4 mL	Immediate post-op:	LRN 4.8 ± 3.7/4 mL **ORN 22.5 ± 26.3/4 mL** LPN 7.9 ± 9.1/4 mL OPN 6.4 ± 6.3/4 mL

CTCn = circulating tumor cell count; **DR** = detection rate; **FU** = follow-up; **LPN** = laparoscopic partial nephrectomy; **LRN** = laparoscopic radical nephrectomy; **OPN** = open partial nephrectomy; **ORN** = open radical nephrectomy.
Bold when statistically significant difference.

correlated with post-op CTCn (r = 0.30, p = 0.01) but not pre-op CTCn. However, TNM staging does not seem to impact CTC release with surgery.(Haga et al., 2020)

4. Discussion

CTCs have the potential to improve our understanding of cancer biology and to become a relevant diagnostic, prognostic, and treatment monitoring biomarker. Liquid biopsies can be repeated to provide real-time information on disease status and molecular profile. However, there is currently not enough evidence to recommend CTC detection in clinical practice in RCC patients. Previous systematic reviews have analyzed the role of CTC in cutaneous melanoma (Rodic et al., 2014), colorectal (Bünger et al., 2015), and pancreatic cancer (Wang et al., 2020), however, to our knowledge this is the first systematic review concerning the use of CTCs in RCC management.

This review identified studies with many limitations, generally with a high risk of bias and a low level of evidence. There are no prospective randomized trials to date and most studies are exploratory. Most designs are observational descriptive and only a few cohort studies have been published, with small and unbalanced samples. Blood sample collection site, timing, number, and volume differed widely. Furthermore, enrichment and detection techniques, as well as molecular markers targeted, varied considerably, hindering comparisons between studies. Hence, no robust conclusions can be drawn. In this review, we chose to include grey literature due to the exploratory phase of this research field. This may be regarded both as an advantage, allowing the inclusion of emerging methods that have not yet been published, and a limitation, since methods and results are frequently interim and cannot be thoroughly analyzed.

Results of CTC DR varied widely between studies and were not consistent between technical platforms and molecular markers. Divergence in results is not fully explained by differences in clinical characteristics and may be associated with the complexity of CTC multi-step processing techniques, causing cell loss and limiting sensitivity.(Hong and Zu, 2013) Additionally, tumor multi-clonality and EMT can change the expression of molecular markers, both in primary tumors and CTCs. (Kasimir-Bauer et al., 2012; Yu et al., 2013; Wu et al., 2015) These phenomena are frequent in RCC and may lead to further CTC subsets loss. To overcome these limitations, the combination of different markers has been attempted, including mesenchymal markers such as vimentin.

Being the only FDA-approved technology for CTC detection, CellSearch® is considered the reference standard in most epithelial cancers, but not in RCC.(Small et al. (2012)) Initial studies targeting EpCAM showed particularly low DR.(Bilkenroth et al., 2001; Meye et al., 2002; Bluemke et al., 2009; Gradilone et al., 2011; Rossi et al., 2012; Glaves et al., 1988) In fact, not all types of CTCs express EpCAM (Hayashi et al., 1999; Engilbertsson et al., 2015) and it has been reported that only 18.6 % of RCC CTCs express this marker(Liu et al., 2016). However, recent studies found a 100 % DR with EpCAM and/or CK using flow cytometry immunofluorescence or size-based enrichment followed by RNA FISH. (Wang et al., 2019; Haga et al., 2020; Ionescu-Zanetti et al., 2015; Tseng et al., 2015) This points to the importance of optimizing both molecular markers and enrichment and detection techniques to maximize performance.

Both CTC presence and CTCn have been shown to correlate with staging, particularly N + and M + status.(Blumke et al., 2005; Liu et al.,

2016; Bluemke et al., 2009; Bai et al., 2018; Hioki and Sugimura, 1999; Shimazui et al., 2003; Liu et al., 2017; Kim et al., 2019; Theil et al., 2014; Haga et al., 2020) Vimentin-expressing CTCs also seem to correlate with more advanced RCC stage.(Liu et al., 2016) However, the role of CTCs as a diagnostic biomarker is still uncertain. It would be interesting to study if CTC presence, quantification, and characterization can be used for RCC risk stratification.

Only six papers correlated CTCs with prognostic outcome measures, with conflicting results. These studies had short follow-ups and outcome measures were not the primary endpoints. Although CTC presence was significantly associated with lower OS in a study by (Bluemke et al. (2009)), two other studies did not confirm this finding (Bai et al., 2018; Gilbert et al., 2006). Concurrently, the impact of CTC detection in PFS was demonstrated in only two of four studies (Nel et al., 2016; Basso et al., 2017). In the single study correlating CTCn with prognostic outcomes, Basso et al. found it had an inverse correlation with OS (Basso et al., 2017). Further studies designed to address the prognostic impact of CTCs are necessary before they can become a part of clinical decision pathways.

A correlation between surgical manipulation and CTC release has previously been demonstrated. In colon cancer, a no-touch isolation technique reduces intraoperative shedding of tumor cells into the portal vein during resection.(Hayashi et al., 1999) In genitourinary cancers, CTC increase after surgery has also been observed in transurethral resection of the bladder (TURB) and brachytherapy patients.(Engilbertsson et al., 2015; Tsumura et al., 2017) In this review, four studies found a CTC increase after surgery,(Bluemke et al., 2009; Ashida et al., 2000; El-Heliebi et al., 2013; Ohlmann et al., 2006) while another study found no difference.(Shimazui et al., 2003) Two research groups investigated post-op CTC DR kinetics and both found a CTCn rise in post-op D1 followed by a decrease after one week, but still above the pre-op value.(Ashida et al., 2000; El-Heliebi et al., 2013) Interestingly, however, two studies revealed CTCs in all patients, both before and after surgery for localized disease.(Wang et al., 2019; Haga et al., 2020) These findings suggest that with a sufficiently sensitive detection method, all patients will present CTCs, which may render CTC DR clinically obsolete. This points towards CTCn as the true biomarker to consider for clinical use. However, CTC persistence in patients who remain relapse-free after radical surgery is still to be explained. Only Wang et al. studied CTC kinetics on longer follow-up. Patients who developed metastases showed an increase in CTCn, unlike patients who remained metastasis-free, who showed a progressive decrease in CTCn (Wang et al., 2019). CTCs may thus be a promising surrogate marker for micrometastases, similarly to serum biomarkers for testicular cancer. Studies with larger samples and longer follow-up are required to test this hypothesis.

Haga et al. were the only ones to compare CTC release among different surgical approaches (Haga et al., 2020). Only ORN showed increased post-op CTCn, unlike LPN, OPN, and LRN on multivariable analysis, although this group had a very significant selection bias towards increased TNM. Properly designed prospective comparative studies are needed to answer the question of whether surgical approach and no-touch protocols can reduce CTC release.

Other candidate RCC biomarkers are being explored, such as serum or urinary microRNA (miRNA), long noncoding RNA (lncRNA), circulating tumor DNA (ctDNA), and other serum or urinary molecules. Some recent exploratory studies have found markers with high sensitivity and specificity, with areas under the curve (AUC) $>$ 0.80 on ROC curve analysis. In an exploratory study, miRNA-15A (miR-15a), a tumor suppressor down-regulated in several cancers, appears to be increased in urinary samples of RCC patients, showing 98.1 % specificity and 100 % sensitivity (AUC 0.96) for RCC versus benign renal masses. (Mytsyk et al., 2018)

Like CTCs, ctDNA offers a real-time non-invasive tool for longitudinal assessment of the patient's cancer genomic profile. Studies have shown a temporal correlation between the detection of specific mutations and progression from localized to metastatic disease. (Bergerot et al., 2018) Pat et al. found that metastatic RCC patients under post-first-line therapies have an increased number of mutations, such as TP53 and VHL, compared to first-line therapy patients (49 vs 25 %), implying a role of specific genomic alterations in therapeutic resistance. (Pal et al., 2017) New Generation Sequencing (NGS) has improved genome analysis in both ctDNA and CTC downstream analysis, allowing whole-genome sequencing (WGS) or probing panels of specific genes or epigenetic alterations associated with a particular cancer.(Mansilla et al., 2018) Beyond its application in therapy guidance, ctDNA detection might also have value in initial cancer diagnosis and detection of early relapse after curative treatment.(Mansilla et al., 2018) Maia et al. detected a significant correlation between ctDNA detection and tumor burden, measured by the sum of the longest diameter of all lesions.(Maia et al., 2017)

Head-to-head studies comparing different biomarkers are lacking. While CTC detection remains technically more challenging compared to ctDNA detection, it provides a single-cell analysis which allows for *in vivo* and *in vitro* cell characterization. ctDNA, on the other hand, has the disadvantage of having multiple sources, hindering whether it is derived from cell death due to effective treatment or persistent tumor cells resistant to therapy.

Combinations of these markers have been explored to improve results, but robust validation studies are still to be published. Some authors have suggested, for instance, the combination of CTC detection and ctDNA analysis in the diagnostic and treatment monitoring settings. (Kidess and Jeffrey, 2013) This may be true because of the known multi-clonality of most cancers, namely RCC, both in the primary tumor, CTCs, and metastatic sites.(Cappelletti et al., 2020)

A clinical RCC biomarker remains elusive. CTCs could be such a biomarker, but so far the lack of robust evidence and technical standardization restricts its clinical use. A sensitive, specific, reproducible, and cost-effective detection technique that can be approved by regulatory agencies is still under investigation.

5. Conclusions

CTCs have the potential to be a diagnostic, prognostic, and treatment monitoring tool in the patient-tailored management era. CTC research in RCC is still in an exploratory phase and no standard detection technique or molecular marker has been found. Current evidence is of low quality, due to highly heterogeneous studies, mostly of retrospective or cross-sectional design and without exploring clinical outcomes. Further research efforts should focus on improving CTC detection accuracy, standardizing CTC definitions and detection methods, and comparing platform performance. Appropriate study designs, with adequate follow-up and clinical outcomes, are required to incorporate these liquid biopsies in clinical decision algorithms for RCC management. Additionally, the combination of different technologies and biomarkers should be explored, in an attempt to achieve panels of biomarkers that can provide a comprehensive insight into both the diagnostic and treatment setting. This is a step needed to enable precision management protocols that can further improve the outcomes of cancer patients.

CRediT authorship contribution statement

Tito Palmela Leitão: had full access to all study data and takes responsibility for its integrity and accurate analysis.

Tito Palmela Leitão: Study concept and design.

Tito Palmela Leitão, Miguel Miranda, Joana Polido, João Morais: Acquisition of data.

Tito Palmela Leitão, Miguel Miranda, Joana Polido, João Morais: Analysis and interpretation of data.

Tito Palmela Leitão, Miguel Miranda, Joana Polido, João Morais: Drafting of the manuscript.

Tito Palmela Leitão, Miguel Miranda, Joana Polido, João

Morais, Tiago Oliveira, Patrícia Corredeira, Patrícia Alves, Ricardo Pereira e Silva, Ricardo Fernandes, João Ferreira, José Palma Reis, Tomé Lopes, Luís Costa: Critical revision of the manuscript for important intellectual content.

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Appendix A. Supplementary data

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8. RESEARCH ARTICLE 2 — PRINTED VERSION: Clinical Validation of a Size-Based microfluidic device for circulating tumor cell isolation and analysis in renal cell carcinoma

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Clinical Validation of a Size-Based Microfluidic Device for Circulating Tumor Cell Isolation and Analysis in Renal Cell Carcinoma

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Abstract: Renal cell carcinoma (RCC) presents as metastatic disease in one third of cases. Research on circulating tumor cells (CTCs) and liquid biopsies is improving the understanding of RCC biology and metastases formation. However, a standardized, sensitive, specific, and cost-effective CTC detection technique is lacking. The use of platforms solely relying on epithelial markers is inappropriate in RCC due to the frequent epithelial-mesenchymal transition that CTCs undergo. This study aimed to test and clinically validate RUBYchip™, a microfluidic label-free CTC detection platform, in RCC patients. The average CTC capture efficiency of the device was 74.9% in spiking experiments using three different RCC cell lines. Clinical validation was performed in a cohort of 18 patients, eight non-metastatic (M0), five metastatic treatment-naïve (M1TN), and five metastatic progressing-under-treatment (M1TP). An average CTC detection rate of 77.8% was found and the average (range) total CTC count was 6.4 (0–27), 101.8 (0–255), and 3.2 (0–10), and the average mesenchymal CTC count (both single and clustered cells) was zero, 97.6 (0–255), and 0.2 (0–1) for M0, M1TN, and M1TP, respectively. CTC clusters were detected in 25% and 60% of M0 and M1TN patients, respectively. These results show that RUBYchip™ is an effective CTC detection platform in RCC.

Keywords: circulating tumor cell; kidney cancer; liquid biopsy; microfluidic; renal cell carcinoma

1. Introduction

Kidney cancer (KC) is the 14th most common malignancy worldwide, with a global incidence of 431,288 in 2020 [1]. The incidence in Europe and North America is considerably higher than in other regions, ranging from 2.09 cases per 100,000 inhabitants (age-standardized rate) in Middle Africa to 24.7 in North America [2]. Renal cell carcinoma (RCC) accounts for the majority (90%) of KC cases [2]. The predominant histological subtypes are clear cell RCC (ccRCC; 70%), papillary RCC (pRCC; 10–15%), and chromophobe

RCC (cRCC; 5%) [2]. The remaining histological subtypes account for less than 1% each [2]. About one-third of RCC patients are diagnosed in metastatic stage and up to 40% of those treated with curative intent relapse and develop metastases during follow-up [3,4].

A clinically useful biomarker is missing for RCC, as diagnosis and follow-up still rely solely on cross-sectional imaging. Several potential biomarkers have been investigated, but none has shown the accuracy and ease of use required for clinical application, particularly for guiding disease management.

The focus of the current research on RCC biomarkers is liquid biopsy. The principle underlying this method is obtaining tumor-derived biological material circulating in the bloodstream through a simple blood sample and accessing phenotypic and genetic data of primary and secondary tumors without the invasiveness of a tumor or metastasis biopsy. This can allow minimally invasive early cancer diagnosis and repeated sequential sampling during disease management to accurately guide treatment decisions, monitor treatment response, and provide prognostic information. Liquid biopsy can focus on a multitude of circulating biomarkers, including circulating tumor DNA (ctDNA), micro RNA (miRNA), and circulating tumor cells (CTCs) [5–8].

CTCs have been a particular focus of interest in liquid biopsy research. The potential clinical value of CTCs has been explored in several tumor types, such as breast, colon, and prostate [9]. Five studies have found a correlation between the presence of CTCs and CTC counts and prognostic outcome measures in RCC, despite their short follow-up and the fact that outcome measures were not primary endpoints [10–14]. Vimentin-expressing CTCs also seem to correlate with more advanced RCC stages [15].

The scientific community is still searching for a sensitive, specific, reproducible, and cost-effective CTC detection technique. CTCs are extremely rare compared to whole blood cells, with estimates indicating one CTC per billion normal blood cells in metastatic disease [16]. CTC enrichment techniques can be classified in four categories: antibody-based (immunomagnetic beads or microfluidics), density-based, size-based (microfluidics and membrane filters), and electrophoresis-based [4]. CTC detection and identification has been accomplished through five different techniques: immunocytochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), cytomorphological criteria, flow cytometry with immunofluorescence, and fluorescence in-situ hybridization (FISH) [4]. Cellsearch® is currently the main CTC detection platform approved by the Food and Drug Administration (FDA) for clinical use and relies on immunomagnetic enrichment and fluorescent labeling for CTC detection [17]. However, it is deemed inappropriate for use in RCC due to the lack of epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) expression in CTCs that have undergone epithelial-mesenchymal transition (EMT), a very common phenomenon in this type of tumor [15]. Only 18.6% of RCC CTCs express EpCAM [15], which explains the particularly low CTC detection rates achieved in initial studies with this marker [18,19].

CTC isolation based on their physical properties is a simpler and more efficient method that relies on differences in cell size and deformability and is independent of molecular markers. However, although more sensitive, it may have lower specificity, given CTC heterogeneity [20]. The principle underlying size-based CTC isolation platforms is the larger size and lower deformability of CTCs compared to blood cells [4]. They have the advantage of minimizing cell loss and allowing downstream analysis of intact cells, but also the limitations of device clogging and some loss of CTCs that are smaller than the device pores. Several authors have used size-based CTC isolation in RCC patients, both membrane-based [21–28] and microfluidic devices [29–31]. Most of these devices are designed to capture cells larger than 8 µm, letting both smaller erythrocytes and deformable leukocytes pass through [32]. Microfluidic-based devices minimize sample processing steps and require shorter processing times, since no sample pre-processing is required [33]. They also require lower reagent volumes and have low contamination issues and sample loss rates. Therefore, cell loss is minimized, especially in samples with low CTC concentration, usually yielding higher sensitivity and detection rates [4].

Due to their potential advantages, microfluidic devices have been investigated as a promising CTC isolation method. RUBYchip™ was shown to be significantly superior to the FDA-approved CellSearch® in CTC isolation in breast, colorectal, gastric, and pancreatic cancer [34–36].

The aim of this study was to test and validate the RUBYchip™ microfluidic device for CTC isolation and analysis in RCC.

2. Results

2.1. CTC Isolation Efficiency

The spiking experiments performed with the RUBYchip™ device resulted in a high RCC CTC yield. The optimal overall capture efficiency was obtained at a 80 µL/min flow rate, enabling the isolation of 77.7%, 77.2%, and 69.8% of spiked Caki-2, A-498, and 786-O cells, respectively (Figure 1). The mean capture efficiency for all RCC cell lines analyzed was 74.9%. At 100 and 120 µL/min, capture efficiency was lower and with higher variability between cell lines, and hence patient samples were subsequently processed at 80 µL/min flow rate.

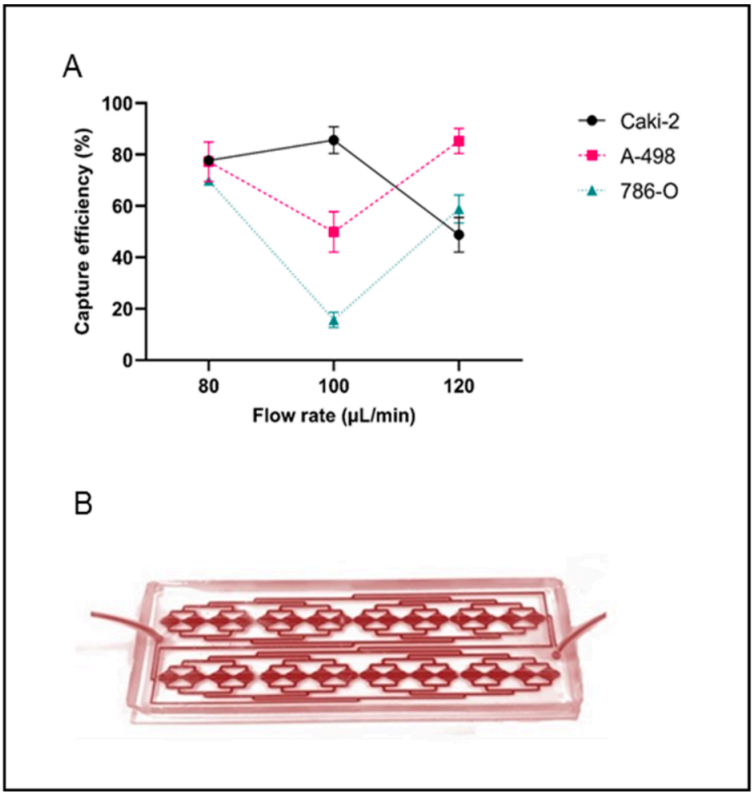

Figure 1. (**A**) Capture efficiency (%) at 80, 100, and 120 µL/min flow rate for Caki-2 (circles with continuous black line), A-498 (squares with dashed red line), and 786-O (triangles with dashed blue line) cells using the RUBYchip™ device. For each flow rate, capture efficiency is represented as the mean and standard deviation (SD) of triplicate experiments. (**B**) RUBYchip™ device running the blood sample of a patient.

2.3. CTC Counts and Characterization

CTCs were detected either as single cells or as clusters of two or more cells (Figure 2). The total CTC count refers to the sum of single CTCs and the number of CTCs in clusters. Two CTC phenotypes were found in all patient groups: epithelial and mesenchymal CTCs. Of note, no EMT CTCs were detected in this cohort.

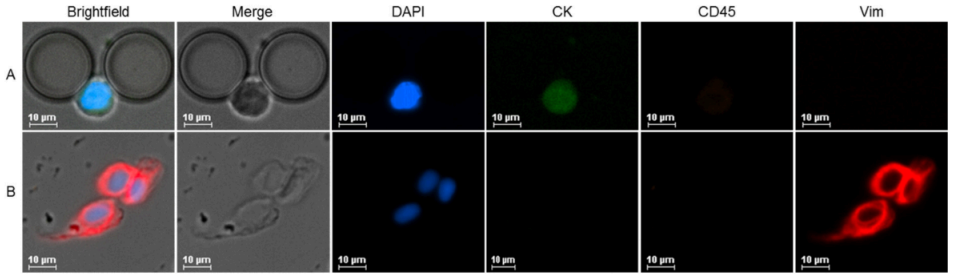

Figure 2. Fluorescence microscopy images at 20X magnification of CTCs from RCC patients captured with RUBYchip™. (**A**) Epithelial CTC (DAPI+/CD45-/CK+/Vim-). (**B**) CTC cluster formed by mesenchymal CTCs (DAPI+/CD45-/CK-/Vim+).

Table 2 and Figure 3 depict CTC counts stratified by phenotype and patient group (M0, M1TN, and M1TP). The detection rate was 77.8% (14/18) overall, 75.0% (6/8) in M0 group, and 80.0% (8/10) in M1 group. CTC clusters were detected in 25.0% (2/8) of M0 patients and in 60.0% (3/5) of M1TN patients. Interestingly, no CTC clusters were detected in M1TP patients. All CTC clusters were composed only of mesenchymal cells.

Table 2. CTC count and phenotype of the study cohort.

	M0			M1TN			M1TP		
	Median	**Average**	**Range**	**Median**	**Average**	**Range**	**Median**	**Average**	**Range**
Single CTCs	1.5	3.3	0–13	49	63.8	0–157	2	3.2	0–10
Epithelial	3	4.2	0–13	0	5.3	0–21	2	3	0–10
Mesenchymal	0	0.0	—	49	59.6	0–157	0	0.2	0–1
CTC clusters	0	0.25	0–1	3	5.8	0–16	0	0.0	—
CTCs in clusters (Mesenchymal)	0	3.1	0–21	31	38.0	0–98	0	0.0	—
Total CTCs *	3	6.4	0–27	80	101.8	0–255	2	3.2	0–10
Epithelial	3	4.2	0–13	0	5.3	0–21	2	3	0–10
Mesenchymal	0	3.1	0–21	80	97.6	0–255	0	0.2	0–1

CTC—circulating tumor cell; M0—localized disease patient group; M1TN—metastatic treatment-naive patient group; M1TP—metastatic progressing-under-treatment patient group. * Total CTCs = single CTCs + CTCs in clusters.

The average (range) total CTC count was 6.4 (0–27), 101.8 (0–255), and 3.2 (0–10) in M0, M1TN, and M1TP groups, respectively. M1TN patients showed a significantly higher number of CTCs than M1TP counterparts (31.8 times higher on average; $p = 0.0003$, 90% CI 30.0–345.6; Figure 4), a difference mainly attributed to the presence of mesenchymal CTCs. The average (range) total (single + clustered) mesenchymal CTC count was 3.1 (0–21), 97.6 (0–255), and 0.2 (0–1) in M0, M1TN, and M1TP patients, respectively, with M1TN patients having significantly more total mesenchymal CTCs than M1TP patients (488.0 times more on average; $p < 0.0001$, 90% CI 31.7–7510.5).

2.2. Characteristics of the Study Cohort

The clinicopathological characteristics of the study's patient cohort are summarized in Table 1 (clinical patient database in Supplementary Material S3). The median age at diagnosis was 60 years for the M0 and M1TP groups and 71 years for the M1TN group. Overall, eight patients (72.7%) had ccRCC, one patient (9.1%) had cRCC, and two patients (18.2%) had pRCC.

Table 1. Clinicopathological characteristics of the study patient cohort.

Clinicopathological Characteristics	Overall	M0	M1TN	M1TP	*p*-Value
Number of patients	18	8	5	5	
Gender, n (%)					
Female	5 (28.0)	2 (25.0)	1 (20.0)	2 (40.0)	1
Age, years					
Median (range)	60 (43–78)	60 (52–70)	71 (43–78)	60 (48–69)	0.511
Smoking habits, n (%)	7 (38.9)	5 (62.5)	1 (20.0)	1 (20.0)	0.252
Obesity, n (%)					
Overweight/Obesity	12 (66.7)	7 (87.5)	1 (20.0)	4 (80.0)	0.05
BMI score (%)					
Median (range)	25.5 (18–38.6)	25.5 (23.6–38.6)	21.5 (21.0–25.4)	25.5 (18.0–27.1)	0.049
Hypertension, n (%)	12 (66.7)	6 (75.0)	3 (60.0)	3 (60.0)	1
Diabetes, n (%)	5 (27.8)	3 (37.5)	1 (20.0)	1 (20.0)	1
ECOG score, n (%)					0.384
0	10 (55.6)	4 (50.0)	3 (60.0)	3 (60.0)	
1	4 (22.2)	3 (37.5)	0	1 (20.0)	
2	2 (11.1)	1 (12.5)	0	1 (20.0)	
3	2 (11.1)	0	2 (40.0)	0	
T stage, n (%)					0.003
T1a	6 (33.3)	6 (75.0)	0	0	
T1b	3 (16.7)	2 (25.0)	1 (20.0)	0	
T2a	2 (11.1)	0	1 (20.0)	1 (20.0)	
T2b	2 (11.1)	0	1 (20.0)	1 (20.0)	
T3a	5 (27.8)	0	2 (40.0)	3 (60.0)	
N stage, n (%)					0.045
N0	13 (72.2)	8 (100.0)	3 (60.0)	2 (40.0)	
N1	5 (27.8)	0	2 (40.0)	3 (60.0)	
Histology, n (%)					0.515
Clear cell	8 (72.7)	4 (80.0)	1 (50.0)	3 (75.0)	
Chromophobe	1 (9.1)	0	1 (50.0)	0	
Papillary	2 (18.2)	1 (20.0)	0	1 (25.0)	
No biopsy (patient preference or unfit)	3	3	3	1	
Metastatic site, n (%)					1
Lung	-	-	3	3	
Bone	-	-	1	1	
Distant lymph nodes	-	-	1	1	
Antiplatelet therapy, n (%)	5 (27.8)	3 (37.5)	2 (40.0)	0	0.416
Systemic therapy, n (%)					
First line	-	-	2	2	
Second line	-	-	-	3	
Unfit for treatment	-	-	3	-	
Treatment, n (%)					
TKI	4 (22.2)	-	1 (20.0)	3 (75.0)	
ICI	3 (16.7)	-	1 (20.0)	2 (40.0)	
Radical nephrectomy	8 (72.7)	5 (62.5)	0	-	
Partial nephrectomy	1 (9.1)	1 (20.0)	0	-	
Surveillance	2 (11.1)	2 (25.0)	0	0	
Unfit for treatment	3 (16.7)	0	3 (60.0)	0	

BMI—body mass index; ECOG—Eastern Cooperative Oncology Group; ICI—immune checkpoint inhibitor; M0—localized disease patient group; M1TN—metastatic treatment-naive patient group; M1TP—metastatic progressing-under-treatment patient group; N—node; T—tumor; TKI—tyrosine kinase inhibitor (according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual Eighth Edition, 2017 [37]). *p*-values concern Fisher's exact test for categorical variables and Kruskal-Wallis test for the quantitative variables.

2.3. CTC Counts and Characterization

CTCs were detected either as single cells or as clusters of two or more cells (Figure 2). The total CTC count refers to the sum of single CTCs and the number of CTCs in clusters. Two CTC phenotypes were found in all patient groups: epithelial and mesenchymal CTCs. Of note, no EMT CTCs were detected in this cohort.

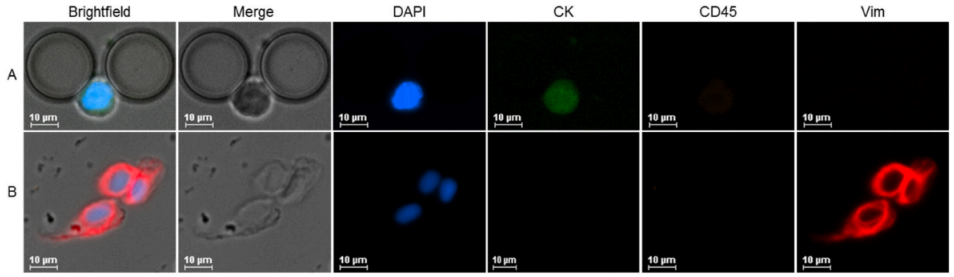

Figure 2. Fluorescence microscopy images at 20X magnification of CTCs from RCC patients captured with RUBYchip™. (**A**) Epithelial CTC (DAPI+/CD45-/CK+/Vim-). (**B**) CTC cluster formed by mesenchymal CTCs (DAPI+/CD45-/CK-/Vim+).

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Epithelial	3	4.2	0–13	0	5.3	0–21	2	3	0–10
Mesenchymal	0	0.0	—	49	59.6	0–157	0	0.2	0–1
CTC clusters	0	0.25	0–1	3	5.8	0–16	0	0.0	—
CTCs in clusters (Mesenchymal)	0	3.1	0–21	31	38.0	0–98	0	0.0	—
Total CTCs *	3	6.4	0–27	80	101.8	0–255	2	3.2	0–10
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CTC—circulating tumor cell; M0—localized disease patient group; M1TN—metastatic treatment-naive patient group; M1TP—metastatic progressing-under-treatment patient group. * Total CTCs = single CTCs + CTCs in clusters.

The average (range) total CTC count was 6.4 (0–27), 101.8 (0–255), and 3.2 (0–10) in M0, M1TN, and M1TP groups, respectively. M1TN patients showed a significantly higher number of CTCs than M1TP counterparts (31.8 times higher on average; $p = 0.0003$, 90% CI 30.0–345.6; Figure 4), a difference mainly attributed to the presence of mesenchymal CTCs. The average (range) total (single + clustered) mesenchymal CTC count was 3.1 (0–21), 97.6 (0–255), and 0.2 (0–1) in M0, M1TN, and M1TP patients, respectively, with M1TN patients having significantly more total mesenchymal CTCs than M1TP patients (488.0 times more on average; $p < 0.0001$, 90% CI 31.7–7510.5).

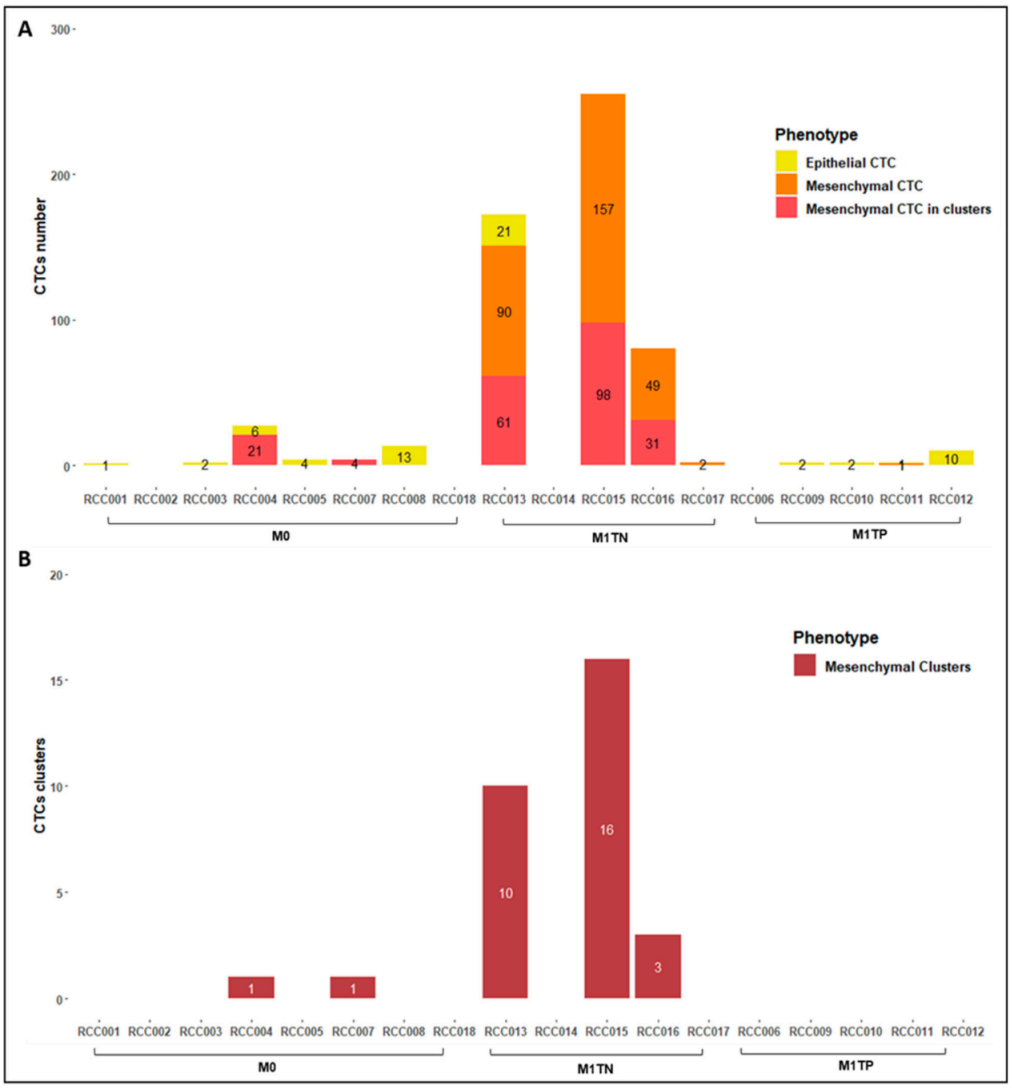

Figure 3. Number of CTCs in M0, M1TN, and M1TP patient groups. (**A**) Number and phenotype of single CTCs and CTCs in clusters. (**B**) Number and phenotype of CTC clusters. Epithelial CTCs are represented in yellow bars, mesenchymal single CTCs in orange bars, mesenchymal CTCs in clusters in light red bars, and mesenchymal clusters in dark red bars.

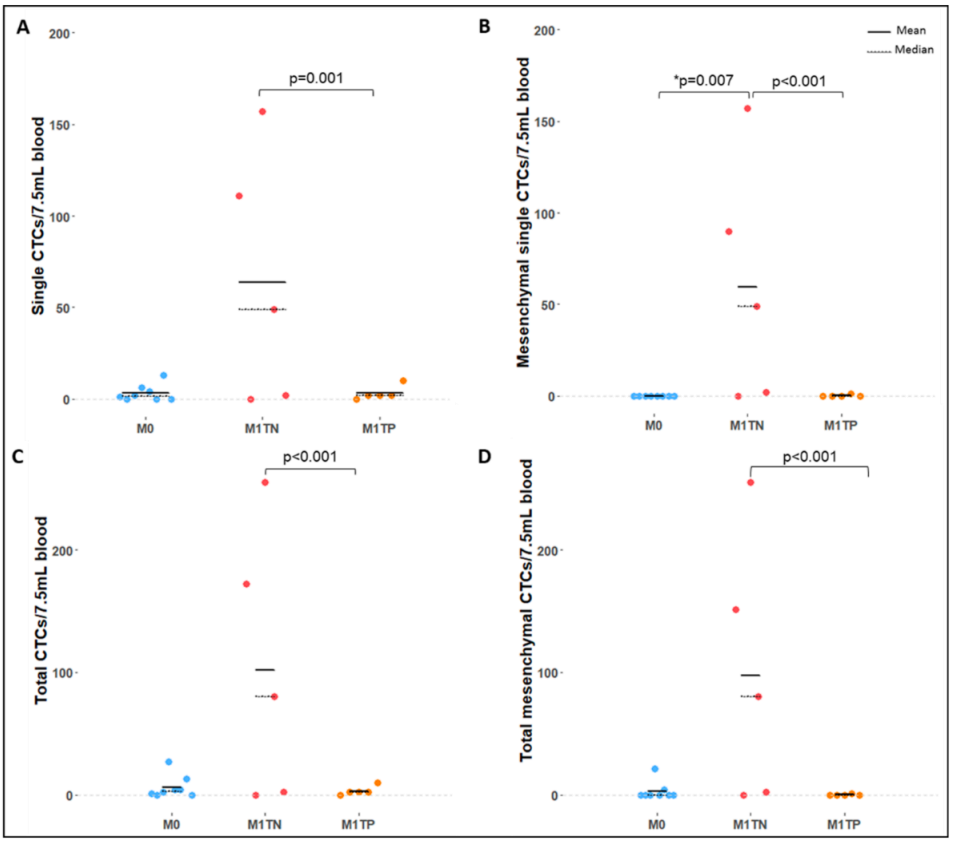

Figure 4. Comparison of CTC counts between M0 (blue dots), M1TN (red dots), and M1TP (orange dots) patient groups. (**A**) Single CTC counts per 7.5 mL whole blood. (**B**) Single mesenchymal CTC counts per 7.5 mL whole blood. (**C**) Total CTC counts per 7.5 mL whole blood. (**D**) Total (single + clustered) mesenchymal CTC counts per 7.5 mL whole blood. Mean represented as a continuous black line and median as a dashed black line. *p*-values obtained via negative binomial regression. * *p*-value obtained through Fisher's test for presence/absence of CTCs.

M1TN patients showed significantly more single mesenchymal CTCs than either M0 ($p = 0.007$) or M1TP ($p < 0.001$) patients. The M1TN group had an average (range) of 59.6 (0–157) single mesenchymal CTCs compared to 0.2 (0–1) in the M1TP group, which means that M1TN patients had 297 times more single mesenchymal CTCs on average than M1TP patients ($p < 0.001$, 90% CI 20.9–4231). Interestingly, no single mesenchymal CTCs were found in the M0 group, although mesenchymal CTCs in clusters were present.

The average (range) single CTC count was 3.3 (0–13), 63.8 (0–157), and 3.2 (0–10) in M0, M1TN, and M1TP groups, respectively. M1TN patients had significantly more single CTCs than M1TP patients (63.8 times more on average; $p = 0.0012$, 90% CI 19.8–205.3). Although more clusters were detected in the M1TN compared to the M0 group, this difference was not statistically significant.

Patients under antiplatelet therapy had significantly more single CTCs ($p = 0.025$), total CTCs ($p = 0.029$), and mesenchymal clusters ($p = 0.031$) compared to patients not receiving that therapy (Figure 5).

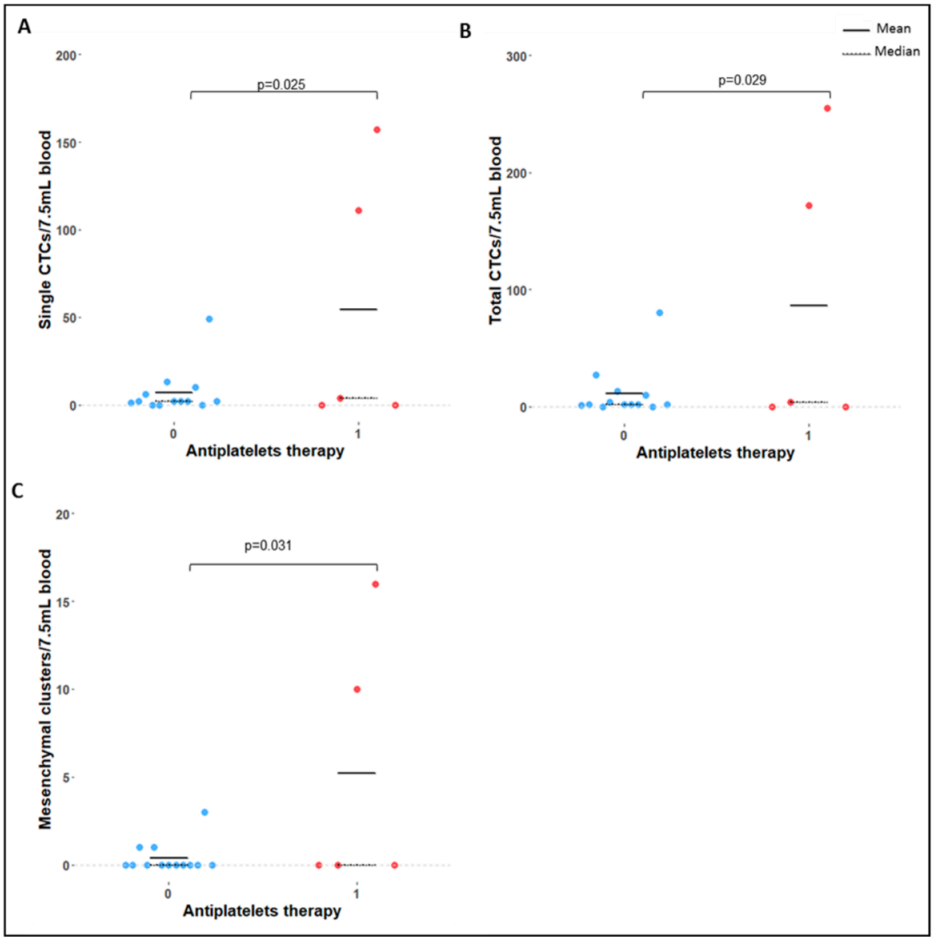

Figure 5. (**A**) Single CTC, (**B**) total CTC, and (**C**) mesenchymal CTC cluster counts in patients. (1) receiving and (0) not receiving antiplatelet therapy. Mean represented as a continuous black line and median as a dashed black line. *p*-values obtained via negative binomial regression.

2.4. Correlation of Clinical Variables with CTC Count and Phenotype

Despite the small number of samples assessed, a strong positive correlation was found between CTC counts and international normalized ratio (INR) in both M0 and M1 groups. In the M0 group, INR correlated with mesenchymal CTCs in clusters and total mesenchymal CTCs (r = 0.85, p = 0.008 and r = 0.85, p = 0.008, respectively). In the M1 group, INR correlated with mesenchymal CTCs (in clusters r = 0.970, p = 0.001, single r = 0.969, p = 0.002, and total r = 0.970, p = 0.002), with CTC clusters (r = 0.975, p = 0.001), with single CTCs (r = 0.974, p = 0.001), and with total CTCs (r = 0.973, p = 0.001).

Interestingly, in the M0 group, a negative correlation was found between epithelial CTCs and leucocyte count (r = −0.748, p = 0.033), while in the M1 group that correlation was positive (r = 0.80, p = 0.005).

In the M0 group, a strong correlation was found between weight and mesenchymal CTCs (r = 0.828, p = 0.011 and r = 0.828, p = 0.011 for mesenchymal CTCs in clusters and total mesenchymal CTCs, respectively) and between body mass index (BMI) and

mesenchymal CTCs (r = 0.878, $p = 0.004$ for both mesenchymal CTCs in clusters and total mesenchymal CTCs).

In the M1 group, increased leukocyte and neutrophil counts also strongly correlated with epithelial CTCs (r = 0.801, $p = 0.005$ and r = 0.852, $p = 0.002$, respectively). A moderate positive correlation was found in the entire cohort and in M1 group between neutrophil-to-lymphocyte ratio (NLR) and total CTC counts, single CTCs, mesenchymal CTCs, and CTCs in clusters.

In M1 patients, serum platelet counts moderately and inversely correlated with total CTCs (r = −0.714, $p = 0.0203$), single CTCs (r = −0.713, $p = 0.0206$), CTC clusters (r = −0.715, $p = 0.0201$), and mesenchymal CTCs in clusters (r = −0.714, $p = 0.0203$), but the same was not observed in M0 patients.

Serum albumin level was moderately and inversely correlated with epithelial CTC counts in M1 patients (r = −0.83, $p = 0.01$), while serum hemoglobin level was moderately correlated with total CTC counts in M0 (r = −0.747, $p = 0.033$) but not in M1 group.

Figure 6 depicts the correlations found in this study between CTC counts and clinical variables assessed.

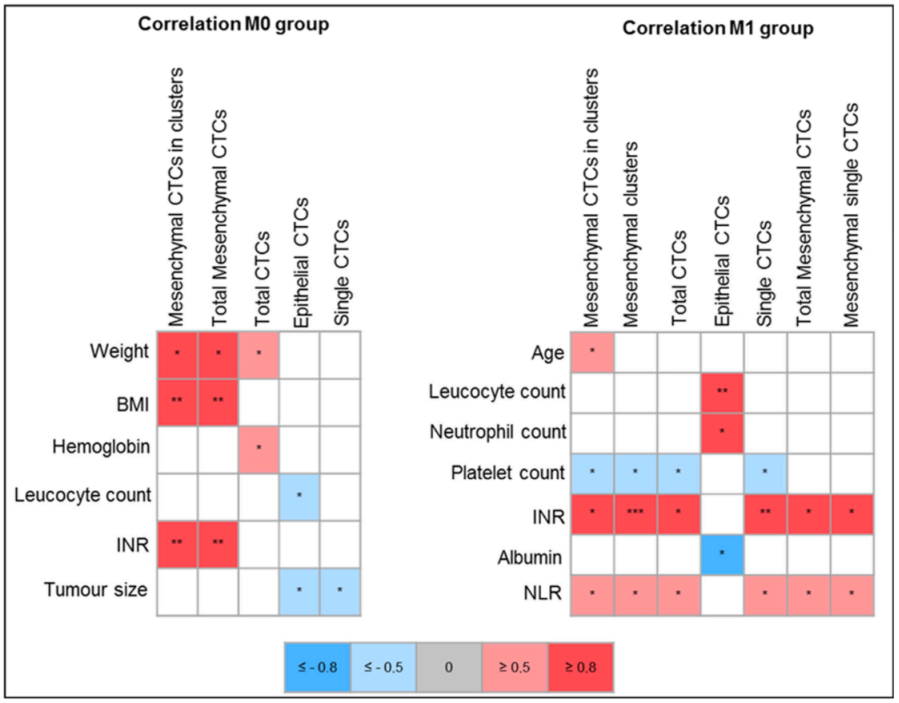

Figure 6. Correlation between CTC counts and clinical variables. BMI—body mass index; CTC—circulating tumor cell; INR—international normalized ratio; M0—localized disease; M1—metastatic disease; NLR—neutrophil-to-lymphocyte ratio. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *p*-values retrieved from Pearson's correlation test. Blue depicts negative correlations: light blue—moderate correlation ($r \geq -0.5$); strong blue—strong correlation ($r \geq -0.8$). Red depicts positive correlations: light red—moderate correlation ($r \geq 0.5$); strong red—strong correlation ($r \geq -0.8$).

The correlation plots can be found on Supplementary Material S1.

2.5. Survival Analysis

Survival analysis is shown in Figure 7. Patients with 5 or more total CTCs had a decreased OS compared to patients with less than 5 CTCs (hazard ratio [HR] 8.45, 95% CI 1.29–55.22; $p = 0.0143$; Figure 7A). This was also true for patients with 5 or more single and total mesenchymal CTCs (HR 7.657, 95% CI 0.717–81.78; $p = 0.0044$; Figure 7C,D) and for those with CTC clusters (HR 0.1306, 95% CI 0.012–1.395; $p = 0.008$). INR, BMI, and NLR did not impact OS. The median follow-up was 11.2 months.

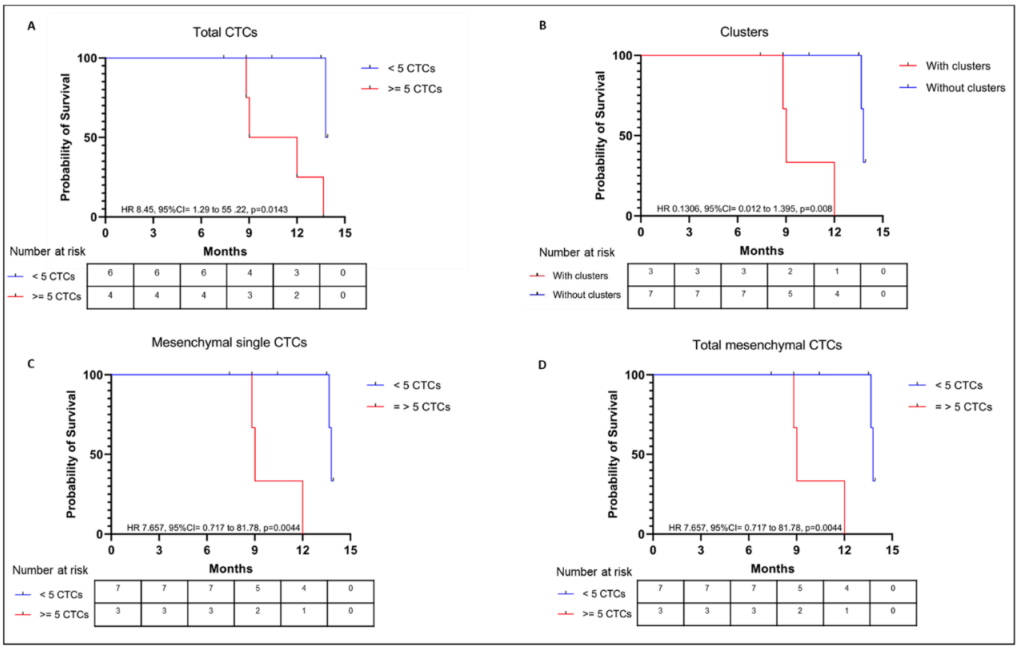

Figure 7. Kaplan-Meyer curves for OS of the metastatic RCC patients' group regarding (**A**) total CTCs, (**C**) single mesenchymal CTCs, and (**D**) total mesenchymal CTCs <5 (blue line) and ≥5 (red line); and for (**B**) presence (red line) or absence (blue line) of CTC clusters.

3. Discussion

3.1. Detection Rates

The mean capture efficiency of RCC cells in RUBYchip™ at the optimal flow rate of 80 µL/min was 74.9%. This high efficiency may be attributed to the absence of whole blood sample preprocessing and to the chip design with prefilters to prevent microclots and obstruction and microfilters to enable white blood cell clearance [36]. The microfilter geometry allows a good balance between the free passage of smaller and/or more deformable cells, like blood cells, and the entrapment of larger and less deformable cells, like CTCs. CTCs are usually larger and less deformable, due to their large nuclei and high cytoplasm-to-nucleus ratio [38]. The wide variability of CTC sizes and phenotypes [39] has prompted the use of three RCC cell culture lines in this study. The results obtained showed consistent detection rates among the three cell lines at the determined optimal flow rate.

In a preclinical validation study of RUBYchip™ conducted in metastatic breast cancer, CTC capture efficiency with this device was up to 10 times higher compared to the CellSearch® system [36]. This was probably because CellSearch® uses preservation tubes, pre-processes blood samples, and only targets EpCAM + CTCs, leading to CTC loss during sample processing.

CTC detection rate in this study's RCC patient cohort was 77.8% overall, reaching 80% in M1 patients and 75% in M0 patients. This is a high detection rate compared to the median of 57% (interquartile range [IQR] 55%) found in a previous systematic review of our group on CTC detection techniques in RCC [4]. RCC is known to have lower CTC detection rates compared to other tumor types, which is thought to be due to a greater prevalence of EMT in this tumor and consequent loss of epithelial markers standardly used to identify these cells [4]. CellSearch® is the first technology approved by the FDA for CTC detection and is regarded as the benchmark in most epithelial cancers, except RCC [40]. It is documented that not all CTCs express EpCAM [41,42], and in the case of RCC, only 18.6% of CTCs seem to express this marker [15].

The similar CTC detection rate achieved in localized and metastatic disease is an interesting finding of this study, as it indicates that most cancer patients have CTCs, even in localized disease stages. Rather than the proportion of patients with CTCs, it is the CTC count that seems to vary with disease stage [4].

3.2. CTC Count

M1TN patients were found to have significantly higher total CTC, single CTC, mesenchymal CTC, and total mesenchymal CTC counts compared to M1TP patients. M1TN patients had 31.8 and 15.9 times more total CTCs than M1TP ($p = 0.0003$) and M0 patients, respectively. These differences are substantial and mainly attributed to the increase in mesenchymal CTCs, with M1TN patients showing 488 and 31 times more total mesenchymal CTCs than M1TP ($p < 0.0001$) and M0 patients, respectively.

Compared to M0 patients, M1TN patients also showed significantly higher total CTC, single CTC, mesenchymal CTC, and total mesenchymal CTC counts, although statistical significance was only achieved for single mesenchymal CTCs ($p = 0.007$).

These findings are in line with other reports in the literature. One other study also reported higher CTC counts in patients with metastatic compared to localized RCC (9.6 vs. 5.3 CTC/7.5 mL) [43]. Liu S. et al. found that CTC counts were 2.2 times increased in late (3 and 4) compared to early (1 and 2) disease stages ($p < 0.001$) [15]. The same authors correlated mesenchymal CTCs with RCC stage. Several other studies have demonstrated a correlation between CTC presence and staging, particularly with N+ and M+ status [10,15,22,29,43–46].

Epithelial CTC counts were very similar among groups, suggesting that disease stage does not have an impact on their number. Interestingly, no EMT CTCs were found in the study cohort. This can be due to the early disease stage of M0 patients, whose cancer cells may not have yet undergone EMT, and/or to the very advanced disease stage of M1 patients, whose transitioning cancer cells may have undergone full epithelial marker loss and concomitant gain of mesenchymal markers, like vimentin.

No significant differences were found in CTC counts between M1TP and M0 patients. This can be a marker of efficacy of systemic therapies in disease control and in limiting CTC release, despite the observed clinical progression. Additionally, no differences were found in CTC counts according to patient characteristics like N stage, smoking, obesity, hypertension, or diabetes. On the other hand, this study was underpowered for the analysis of ECOG score, T stage, and tumor histological subtypes.

Most CTCs in the M1TN group were found to be mesenchymal and only one M1 patient presented epithelial CTCs, which is in line with the relevant EMT known to occur in advanced RCC. Although the metastatic process is not yet fully understood, it is generally accepted that EMT plays a role in CTC release and is an important factor explaining tumor progression and treatment resistance [47]. A link between EMT and disease aggressiveness, treatment response, and survival has already been established in several tumor types [48,49].

In this study, mesenchymal CTCs were defined as DAPI+/CD45-/CK-/Vim+ cells. However, some controversy exists in this definition. Vimentin is the most used marker for mesenchymal phenotyping, but other markers, like N-cadherin, O-cadherin, fibronectin,

serpin peptidase inhibitor, and twist have been studied [50]. However, no marker or panel of markers has been identified as being able to definitely identify EMT or mesenchymal CTCs to date. Further characterization of these CTC subsets, for instance with downstream analysis using DNA and RNA sequencing, may improve the understanding of EMT and the role of these cells in cancer progression [51]. It has been proposed that some vimentin-positive cells could be circulating cancer-associated fibroblasts (cCAF) [52]. It has also been reported that metastatic CTCs are more viable when integrated into heterotypic clusters consisting of tumor and stromal cells [53]. Spindle-shaped vimentin-positive cells were identified in some samples in this study and considered as possible cCAF and not counted as CTCs. Only vimentin-positive CTCs with specific cytomorphological features, like round/ovoid shape, big nuclei, and high nucleus-to-cytoplasm ratio have been considered.

It should be noted that the first FDA-approved technology for CTC capture and analysis in clinical setting in most tumors—CellSearch®—only relies on the expression of epithelial markers, which has hindered RCC CTC research in the context of the widely present EMT in this tumor type. Hence, CTC isolation platforms capable of detecting EMT, mesenchymal CTCs, and CTC clusters, like the one used in this study, should be employed in future RCC research to clarify the clinical significance of these CTC subpopulations, and further elucidate the biology of kidney cancer. In 2022, the Parsortix® microfluidic platform has also received FDA approval for CTC detection in metastatic breast cancer, confirming microfluidics has a promising technology in cancer.

3.3. CTC Clusters

CTC clusters can be defined as a group of 2–3 to 100 cancer cells [50,54]. Aceto et al. reported that CTC clusters had 23–50 times more metastatic potential than single CTCs, despite representing only 2–5% of all CTC events detected in a breast cancer mouse model [54]. Animal models have demonstrated that CTC clusters arise from primary tumor vein invasion and fragmentation rather than aggregation of single CTCs [55]. It has also been shown that the injection of clustered cells resulted in reduced OS in mice compared to the injection of single CTCs (12.7 vs. 15.7 weeks, $p < 0.016$) [54]. In the same mouse model, CTC clusters were cleared from circulation at least three times more rapidly than single CTCs (half-life: 6–10 min for CTC clusters vs. 25–30 min for single CTCs) [54].

In the present RCC patient cohort, a higher average CTC cluster count was found in M1TN compared to M0 and M1TP patients. Although these differences were not statistically significant (possibly due to the small sample size), they seem clinically relevant, particularly in the M1TP group, where no clusters were found, suggesting efficacy of systemic treatment in preventing CTC cluster formation and release. Interestingly, all CTCs in these clusters were mesenchymal, in agreement with other data in the literature reporting that CTC clusters seem to be more frequently composed of mesenchymal rather than epithelial CTCs [51].

3.4. CTCs and Survival Outcomes

Patients with 5 or more total CTCs, with mesenchymal CTCs (both single and total), and with CTC clusters were found to have significantly lower OS in this study. Several other studies had previously documented the impact of the presence of CTCs and of CTC counts on RCC survival [4,10,12,13,56–58]. One study showed that patients with CTC counts with >0.12 CTCs/mL annually, had shorter OS compared to patients with <0.12 CTCs/mL (median 17.0 vs. 21.1 months, $p < 0.001$) [56]. In another study, patients with mesenchymal CTCs had a slight survival decrease compared to patients without these cells (HR 1.2, 95% CI 1.1–1.4; $p = 0.005$) [59]. Another group found that total postoperative CTC counts higher than 6, presence of postoperative mesenchymal CTCs, and presence of postoperative CTC-white blood cell clusters significantly correlated with recurrence and metastases [57]. In a study in M1 RCC, patients with total CTC counts >3 had shorter OS than patients with $\leq$3 (median 13.8 vs. 52.8 months on multivariate analysis; HR 1.67, 95% CI 0.95–2.93;

$p = 0.003$) [13]. A standard CTC count cut-off to predict prognostic outcomes is yet to be determined.

Similar findings were reported in other tumor types. In colorectal cancer, patients with more than 3 CTCs/7.5 mL had reduced survival [60,61]. The risk of tumor progression and death was higher in CTC-positive patients with pancreatic and esophageal cancer [62,63]. OS also correlated with high CTC counts in a meta-analysis of gastric cancer [64].

3.5. Correlation of CTC Counts with Clinical Variables

A very strong positive correlation was found between all CTC counts and INR in metastatic patients, with the correlation being more moderate in patients with localized disease. This could be explained by the acknowledged prothrombotic state elicited by CTCs and cancer in general, which may cause coagulation factor consumption and increased INR [65]. The EMT process can cause overexpression of tissue-factor in CTCs, conferring procoagulant properties that can contribute to metastases formation [65,66]. However, in the study by Dirix and colleagues, no significant association was found between activated partial thromboplastin clotting time or prothrombin time and CTC counts [67].

Patients' weight and BMI showed a strong positive correlation with mesenchymal CTC counts in the M0 group. Age showed a moderate positive correlation, consistent with all CTC counts. It can be hypothesized that age may hinder immunity, which, together with the general patient frailty, may promote CTC survival.

Since CTCs in circulation interact with other blood cells and components, a possible relation between CTC counts and other blood constituents was investigated.

Leukocyte counts were found to have a moderate positive correlation with epithelial CTCs in M1 patients, but a negative correlation in M0 patients. The latter was only observed for epithelial CTCs and not for mesenchymal CTCs or clusters. Tumor-associated neutrophils appear to contribute to CTC survival by suppressing peripheral leukocyte activation in advanced cancer patients [68]. Additionally, single CTCs have been shown to have impaired interactions with T lymphocytes and natural killer (NK) cells, being this way protected against recognition by the immune system [69]. On the other hand, heterotypic CTC clusters have increased aggressiveness, namely when CTCs are conjugated with platelets, leukocytes, neutrophils, tumor-associated macrophages, and fibroblasts [69]. In addition, metastasis-promoting gene expression profile changes were shown to occur with CTC and neutrophil interaction in a breast cancer mouse model study [69].

NLR positively correlated with total CTCs, single CTCs, mesenchymal CTCs, and CTC clusters in this cohort, particularly in metastatic patients. In M1 group, increased neutrophil counts also strongly correlated with epithelial CTCs, but not with other CTC variables. In a study by Peyton et al., an increase in absolute neutrophil count and NLR >4 were independent predictors of decreased survival in RCC ($p < 0.05$) [70]. In stage II/IV gastric cancer, CTC detection was also significantly correlated with neutrophil count ($p = 0.020$) and NLR ($p = 0.009$) [71]. Therefore, NLR and neutrophil counts may prove to be surrogate predictors of survival in RCC.

In the present study, C-reactive protein (CRP) levels positively correlated with epithelial CTC counts, suggesting that an elevation in inflammatory parameters may correlate with increased CTC count. In a study in ovarian cancer, CRP was higher in CTC-positive versus -negative patients, with a median of 4.33 (IQR 1.46–7.51) versus 1.52 (IQR 0.50–4.50), respectively ($p = 0.001$) [72]. CRP has been shown to have prognostic value in predicting outcomes, as well as the ability to predict response to chemotherapy in various tumor types [73]. A recent study demonstrated a strong correlation in RCC between coagulation and both CRP and CTCs [74].

In this study's cohort, serum platelet count was inversely correlated with total CTC, single CTC, and CTC cluster counts, but only in metastatic patients. Some studies have suggested that activated platelets can shield CTCs and protect them from immune destruction and blood flow shear forces [75–77]. CTC-coating platelets can produce major histocompatibility complex I-positive vesicles that may help CTCs to escape recognition

by NK and T cells [78]. This platelet recruitment and activation could lead to platelet consumption, decreasing their serum counts, which would help explain the inverse relation found in this study. A 2022 paper by Dirix et al. also found a negative correlation between platelet count and CTC count in advanced breast cancer ($p < 0.0009$, R2 0.167) [67]. On the other hand, Guan et al. found a positive correlation between mesenchymal CTCs and platelet levels in RCC [59].

Platelet interaction with CTCs may also lead to EMT induction and maintenance through TGF-ß release, thereby promoting metastases formation [79]. This suggests that platelet action may promote CTC survival and metastases, but further studies are required to clarify the relation between platelet and CTC counts.

Serum hemoglobin levels were found to correlate with total CTC counts moderately and inversely in the M0 group. In a study in prostate cancer, a negative association was found between CTC counts and hemoglobin levels ($p = 0.004$) [80], and other studies have confirmed this association [81,82]. Also, serum albumin showed a moderate and inverse correlation with CTC counts in M0 patients, but only with epithelial CTC counts. In this cohort, patients with more advanced disease and higher tumor burden had poorer performance status and concomitantly lower levels of albumin and higher levels of CRP. Hypoalbuminemia is a surrogate marker of known disease processes present in advanced cancers, like increased catabolism, systemic inflammatory response, increased vascular permeability and interstitial edema, and decreased albumin synthesis [83]. The correlation of serum albumin and CTCs is poorly studied. In a study in ovarian cancer, no differences were found in serum albumin levels between CTC+ and CTC- patients [72].

3.6. Final Remarks, Study Limitations and Future Directions

In sum, this study showed that the RUBYchip^{TM} consistently detected CTCs in distinct groups of patients suffering from RCC irrespective of CTC counts (low vs. high), phenotype (mesenchymal vs. epithelial) and degree of aggregation (singlets vs. clusters). In treatment-naïve patients with metastatic disease (M1TN group) we consistently found increased total CTCs, namely single CTCs, chiefly contributed by the mesenchymal phenotype.

The main limitation of this study is its small sample size, which made it underpowered for several analyses. Despite the limited clinical conclusions that can be drawn from such a small patient cohort, a positive correlation was identified between CTC counts and both staging and prognosis.

The future of liquid biopsy in cancer is promising, being generally agreed that they will play a crucial role in cancer diagnosis, treatment, and monitoring in upcoming years. Some of the advantages of liquid biopsies are its non-invasive nature, real-time monitoring potential, and ability to provide a comprehensive picture of cancer cells and their behavior.

However, there are still challenges ahead, such as the need for standardization and improved accuracy of liquid biopsy testing. Nevertheless, research in the field is ongoing, and it is predictable that liquid biopsy technology will continue to move forward and become an increasingly important tool in the fight against cancer.

Further investigation is required to identify effective molecular markers and develop reliable, standardized techniques for isolation and detection of CTCs in RCC, so that they can be used as diagnostic, prognostic, and treatment management tools.

4. Materials and Methods

4.1. Microfluidic Device

The RUBYchip™ device (RUBYnanomed/International Iberian Nanotechnology Laboratory [INL], Braga, Portugal, PCT/EP2016/078406) is a microfluidic system that captures CTCs from whole blood samples based on cell size and deformability [36]. The device consists of an inlet that directs the sample through a network of interconnected capillaries into multiple cell-filtering chambers. Each chamber has transverse rows of micropillars that make up the cell filtering area. The size, geometry, and gap size of the pillars were designed so that deformable white blood cells gently flow through, while larger, more rigid

cells, like CTCs, are retained in the cell-filtering chamber. The fabrication process, technical specifications, and details of the device are described elsewhere [36].

4.2. Cell Culture

Human KC cell lines Caki-2 (ATCC, HTB-47), A-498, and 786-O were used for spiking experiments. The Caki-2 cell line was cultured in McCoy's 5A Medium (Gibco™, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and the A-498 cell line in Dulbecco's Modified Eagle Medium (Gibco™), and 786-O in Roswell Park Memorial Institute 1640 (Gibco™). All growth media were supplemented with 10% fetal bovine serum (Gibco™) and 1% penicillin/streptomycin (Gibco™). All cell lines were maintained at 37 °C with 5% CO_2 in a humidified atmosphere, at a low passage, and routinely tested for mycoplasma contamination by quantitative polymerase chain reaction (GATC Biotech, Konstanz, Germany).

4.3. Spiking Experiments

The capture efficiency of the RUBYchip™ device in RCC was assessed through spiking experiments using Caki-2, A-498 and 786-O cell lines. Approximately 200 cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI) (10 µg/mL, Sigma-Aldrich, Burlington, MA, USA) after trypsinization and added to 7.5 mL of whole blood samples from healthy donors. To find the best conditions, samples were injected in RUBYchip™ using a syringe pump (KF Technology) at three different flow rates: 80, 100, and 120 µL/min. Afterwards, devices were washed with 2% bovine serum albumin (BSA) (NZYtech Lda, Lisbon, Portugal) in phosphate-buffered saline (PBS) 1X, fixed with 4% formalin for 20 min at room temperature and finally washed with 0.5% BSA in PBS 1X followed by 1% sodium azide (Sigma Aldrich) in PBS 1X. As previously described, cell counting control of the spiked cell number was performed by pipetting the same cell suspension volume into a well plate [36]. Fluorescence cell images were acquired using an inverted fluorescence Nikon Eclipse Ti microscope at 20× magnification. Experiments were performed in triplicate. Capture efficiency was calculated as the ratio between the number of DAPI-positive cells trapped inside the device and the average cell count in the well plate, as previously described by Ribeiro-Samy S, et al. [35]:

$$CTC\ capture\ efficiency\ (\%) = \frac{Trapped\ cells}{Spiked\ cells} \times 100\ (\%) \quad (1)$$

4.4. Immunocytochemistry Protocol and Immunofluorescence Imaging

Different experimental conditions were tested to optimize the antibody staining protocol in the cells trapped in the spiking experiments. The selected antibody panel included AF647 anti-human vimentin (Biolegend, 1:50, San Diego, CA, USA), phycoerythrin-conjugated anti-human CD45 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA, 1:50), and DAPI (1 µg/mL). Two antibodies were tested to stain cytokeratin: anti-human cytokeratin 8/18 unconjugated ready-to-use antibody (Dako, Agilent, 200 µL, Santa Clara, CA, USA), detected with FITC goat anti-rabbit IgG cross-adsorbed secondary antibody (Invitrogen, Thermo Fisher Scientific, 1:1000); and FITC-conjugated anti-human cytokeratin (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). After isolation, cells were permeabilized with 0.25% triton X-100 for 10 min, then rinsed with PBS 1X. The antibody panel was incubated for 1 h at room temperature in the dark after a blocking step with 2% BSA in PBS 1X for 20 min. Samples incubated with unconjugated cytokeratin antibody were subsequently incubated with the secondary antibody for 30 min at room temperature in the dark and washed with 0.5% BSA in PBS 1X and 1% sodium azide in PBS 1X. Images were obtained using an inverted fluorescence Nikon Eclipse Ti microscope at 20× magnification.

4.5. Patient Recruitment and Sample Collection

To validate RUBYchip™ for clinical use in RCC, 18 patients were enrolled at Centro Hospitalar Universitário Lisboa Norte (CHULN), Lisbon, Portugal, between August 2021 and May 2022. Patients were divided into three groups: a localized disease group (M0 group) with eight patients, whose samples were collected prior to treatment with curative intent; and a metastatic disease group (M1 group) of 10 patients, five of which were treatment naïve (M1TN group; n = 5,) and the remaining five were diagnosed with disease progression under systemic therapy (M1TP group; n = 5). Progression was defined according to RECIST criteria [84]. The study was approved by the Ethics Committee of CHULN and conducted in accordance with the Declaration of Helsinki and good clinical practice guidelines. All patients provided and signed informed consent before any study procedure. Objective tumor status was assessed through Tumor, Node, Metastasis (TNM) criteria [37]. Single 7.5-mL peripheral blood samples were collected in EDTA tubes at the time of diagnosis in M0 group, before systemic therapy in M1TN group, and after tumor progression in cross-sectional imaging follow-up in M1TP group. All samples were anonymized, and a code was assigned before sample processing.

4.6. CTC Isolation and Characterization

Clinical samples were processed in the RUBYchipTM device within 40 min after collection. The 7.5-mL blood samples were injected at 80 µL/min, and the CTCs entrapped in the device were then washed and fixed, as described in the spiking experiments (Section 2.3). CTCs were stained inside the device with the previously described antibody panel (Section 2.4) and under the same conditions. Samples from the first 12 patients were stained with the anti-human unconjugated cytokeratin 8/18, and those from the last six patients with FITC-conjugated anti-human cytokeratin 8/18.

After image acquisition, cells were manually enumerated and classified by randomized blind analysis performed by two independent operators. No variability was found in the cytokeratin signal with the two antibodies, but a reduction in the background FITC signal was observed with the conjugated antibody. Cells were identified as CTCs and distinguished according to their phenotype using the following criteria: epithelial CTCs were DAPI+/CD45-/CK+/Vim-, mesenchymal CTCs were DAPI+/CD45-/CK-/Vim+, and EMT CTCs were DAPI+/CD45-/CK+/Vim+ [35,36,85]. In addition, cells had to show membrane integrity in brightfield, a round nucleus, and cell-like morphology to be classified as CTCs. CTC clusters were defined as groups of two or more cells characterized by having regular contours, being in close contact with each other, and complying with the above criteria [54]. A CTC counting matrix was used to enumerate the difference CTC subsets (Supplementary Material S2)

4.7. Statistical Analysis

Continuous variables were presented as median, average, and range, and categorical variables as absolute and relative frequencies. Fisher's exact test was used to compare the three patient groups (M0, M1TN, and M1TP) for the presence/absence of CTCs. Negative binomial regression was used to compare groups regarding CTC counts, including M1TN versus M1TP groups, pathological lymph node presence, smoking habits, hypertension, diabetes, overweight/obesity, and antiplatelet therapy. Pearson's correlation was used to assess the correlation between CTC counts and quantitative clinical variables. Overall survival (OS) was defined as the time from sample collection to metastatic patients' (M1TN and M1TP) last clinical follow-up or death and estimated using the Kaplan-Meier estimator. Median OS and 95% confidence interval (CI) were computed. Results were considered statistically significant if the p-value was less than 0.05. Statistical analyses were performed with R Software v2022.07.1 (R Foundation for Statistical Computing, Vienna, Austria). Survival analysis was performed using GraphPad Prism 8 v8.4.3 (686).

5. Conclusions

The findings of this study show that the RUBYchip™ microfluidic size-based CTC detection device is an effective and reproducible method for isolating CTCs in RCC. It has high detection rates with short processing times due to fewer processing steps compared to other devices. It is able to identify different CTC phenotypes and detect CTC clusters, which are relevant in this tumor type. The RUBYchip™ can thus be used in future RCC research to help improve the understanding of the metastatic process and disease progression, as well as to potentially guide patient management.

6. Patents

The RUBYchip™ is based on patent PCT/EP2016/078406, filed by INL in front of EPO on 22 November 2016, covering the geometry and surface coating of the microfluidic system for CTC isolation, and currently licensed exclusively to RUBYnanomed.

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24098404/s1.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in the Supplementary Materials.

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Conflicts of Interest: The exploitation rights of the above-mentioned patent have been licensed to the spin-off company RUBYnanomed, incorporated by some of the authors.

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9. RESEARCH ARTICLE 3 — PREPRINT VERSION: Circulating tumor and mesenchymal cell release in no-touch radical nephrectomy: a randomized controlled trial

Circulating tumor and mesenchymal cell release in no-touch radical nephrectomy: a randomized controlled trial

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Article

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Abstract

Introduction:

Circulating tumor cells (CTCs) may be the missing renal cell carcinoma (RCC) biomarker.

Material and Methods:

Randomized controlled trial comparing CTC and circulating mesenchymal cell (CMC) release in no-touch (NT) vs. conventional (C) laparoscopic RN. Blood samples were collected at operation room arrival (S0), specimen extraction (S1), postoperative D1, and D30. CTCs isolated and analyzed using the RUBYchip™.

Results:

34 patients were randomized from September 2021 to April 2022. No differences were found in CTC and CMC counts, count variations between time points, complications, and outcomes between groups. The total circulating cell detection rates in the NT, C, and overall RCC groups were 58.3%, 80.0%, and 70.4% at S0, 41.6%, 86.7%, and 66.7% at S1, 50.0%, 64.3%, and 60.0% at D1, and 54.5%, 42.9%, and 44.0% at D30, respectively. A progressive decrease in CMCs was observed after surgery in the C group, mainly at D1 (4.78 to 1.64 CMCs/7.5mL-blood, p = 0.035). Healthy controls showed no circulating cells. High CMC counts were found in chronic inflammation controls and oncocytoma patients, not significantly different from RCC patients.

Conclusions:

NT RN did not reduce circulating cell release nor improve survival.

1. INTRODUCTION

Renal cell carcinoma (RCC) had a global incidence of 431,288 in 2020[1]. Surgery is the preferred treatment for localized disease, although recurrence occurs in 20–40% of cases[2]. Radical nephrectomy (RN) is performed in half of the cases, whenever a partial nephrectomy is not possible[3]. Despite early detection significantly increasing in recent years, a third of RCC patients still present with metastatic disease[1,4].

Circulating tumor cells (CTCs) have been described as a potential RCC biomarker[5]. There is no clinically validated RCC biomarker currently available. CTCs have been shown to correlate with staging and survival in RCC[6–8]. Epithelial cell-adhesion molecule (EpCAM)-based CellSearch® was the first FDA-approved platform for CTC analysis, providing prognostic information in metastatic breast, prostate, and colorectal cancer[9]. However, the fact that only 18.6% of RCCs express EpCAM raised the need for alternative approaches for CTC analysis in RCC[6]. Maertens and colleagues reported that a cell-size-based system was the most efficient CTC isolation platform in RCC cell lines[10].

Exploratory studies of perioperative CTC kinetics in RCC revealed an increase in counts at D1 and a decrease on subsequent days[11–14]. Bluemke *et al.* found that pre-operative CTC detection had a relative risk of death of 2.7 (p = 0.049), and postoperative detection of 4.3 (p = 0.036)[13]. These results suggested that intraoperative tumor manipulation increases CTC release in RCC surgery and impacts prognosis.

The concept of no-touch (NT) tumor resection was first introduced in 1977 in colorectal cancer[15]. The NT group had less RT-PCR tumor mutations detection compared to those patients operated on using a conventional (C) technique (73% vs. 14%, respectively, p = 0.05)[16]. However, two RCTs failed to demonstrate a difference in outcomes for NT resection in colon cancer[17,18]. In a prospective lung cancer trial, CTC detection after surgery was significantly lower in an NT compared to a C approach (12.5 vs. 85.7%, p = 0.02, respectively)[19].

RN was classically performed according to the five 1963 Robson principles[20]. All have been rebutted except for early renal pedicle ligation. A few publications described this technique as safe, although with no benefit in outcomes[21–23]. To our knowledge, there are no publications addressing the NT technique for RN.

The objective of this study was to investigate whether surgical manipulation of the kidney during RN increases circulating cell (CC) release and whether it can be reduced during RN by using an NT technique. The association between CC release and patient prognosis was a secondary objective.

2. RESULTS

Thirty-four patients were randomly assigned to the NT (n = 18) and C (n = 16) groups (Fig. 1).

Baseline clinicopathological characteristics of the study participants and controls are presented in Table 1.

Table 1
Clinicopathologic characteristics of the study population according to intervention groups and study controls

	NT group n = 16	C group n = 16	$p^{\dagger}$	Control group n = 9	$p^{\ddagger}$
Age at surgery (yr), median (quartiles)	61.5 (56.8–70.5)	61.0 (55.8–71.0)	0.985	65 (55.0–69.0)	0.912
Gender M, n (%)	9 (56.25%)	13 (81.25%)	0.252	5 (80%)	0.693
BMI (kg/m2), median (quartiles)	26.2 (23.6–30.1)	26.9 (24.1–31.4)	0.509	29.3 (27.0–30.1)	0.637
Weight (kg), median (quartiles)	76.0 (68.8–88.5)	79.0 (69.5–98.5)	0.706	80.0 (77.5–91.0)	0.517
Height (cm), median (quartiles)	170.5 (161.2–177.0)	170.5 (166.8–176.8)	0.925	180.0 (170.0–181.0)	0.461
Smoking, n (%)	6 (37.5%)	4 (25%)	0.704	3 (33.3%)	1
Obesity, n (%)	5 (31.3%)	5 (31.3%)	1	2 (22.2%)	0.702
Hypertension, n (%)	11 (68.8%)	13 (81.3%)	0.685	6 (66.7%)	0.680
Diabetes, n (%)	2 (12.5%)	1 (6.25%)	1	4 (44.4%)	0.031
Antiplatelet Therapy, n (%)	5 (31.3%)	5 (31.3%)	1	2 (22.2%)	0.702
Anticoagulation, n (%)	1 (6.25%)	5 (31.3%)	0.172	0	0.309
ECOG, n (%)			0.654		0.028
0	6 (37.5%)	8 (50%)		2 (22.2%)	
1	7 (43.8%)	4 (25%)		5 (55.6%)	
2	3 (18.8%)	4 (25%)		0	
3	0	0		2 (22.2%)	
GFR (mL/min/1.73), median (quartiles)	64.5 (38.3–81.25)	81.5 (61.8–97.0)	0.239	73.0 (54.0–90.0)	0.935
Stage 1 (> 90 mL/min), n (%)	3 (18.8%)	6 (37.5%)		3 (33.3%)	
Stage 2 (> 60–89 mL/min), n (%)	6 (37.5%)	6 (37.5%)		2 (22.2%)	
Stage 3 (> 30–59 mL/min), n (%)	4 (25%)	1 (6.25%)		3 (33.3%)	
Stage 4 (> 15–29 mL/min), n (%)	1 (6.25%)	2 (12.5%)		1 (11.1%)	
Stage 5 (< 15 mL/min), n (%)	2 (12.5%)	1 (6.25%)		0	
CKD History, n (%)			0.273		1
0	8 (50%)	11 (68.8%)		5 (55.6%)	
1	8 (50%)	4 (25%)		4 (44.4%)	
2	0	1 (6.3%)		0	
Dialysis, n (%)	3 (18.8%)	1 (6.3%)	0.600	0	0.559
Autoimmune disease, n (%)	3 (18.8%)	1 (6.3%)	0.600	0	0.559
Tumor characteristics:					
cT			1		1
cT0, n (%)	3 (18.8%)	2 (12.5%)		0	
cT1b, n (%)	5 (31.3%)	6 (37.5%)		0	
cT2a, n (%)	3 (18.8%)	4 (25%)		0	
cT2b, n (%)	2 (12.5%)	1 (6.3%)		0	
cT3a, n (%)	2 (12.5%)	3 (18.8%)		0	
cT3b, n (%)	1 (6.3%)	0		0	
cN			1		1
cN0, n (%)	16 (100%)	15 (93.75%)		0	
cN1, n (%)	0	1 (6.3%)		0	
cM			0.484		1

	NT group n = 16	C group n = 16	$p^{\dagger}$	Control group n = 9	$p^{\ddagger}$
M0, n (%)	16 (100%)	14 (87.5)		0	
M1, n (%)	0	1 (6.3%)		0	
Mx, n (%)	0	1 (6.3%)		0	
Tumor side			0.716		0.039
Right, n (%)	11 (68.8)	9 (56.3%)		9 (100%)	
Left, n (%)	5 (31.3%)	7 (43.8%)		0	
Tumor size (mm), median (quartiles)	59.5 (40.8–98.3)	59.0 (49.0–75.5)	0.865	NA	NA
Peri operative characteristics:					
ASA, n (%)			0.767		1
I	1 (6.3%)	1 (6.3%)		0	
II	7 (43.8%)	6 (37.5%)		0	
III	8 (50%)	7 (43.8%)		0	
IV	0	2 (12.5%)		0	
Blood loss (ml), median (quartiles)	100.0 (50.5–212.5)	90.0 (50.0–375.0)	0.849	NA	NA
Peri operative blood transfusion, n (%)	0 (0)	0 (0)	0.317	NA	NA
Operative time (min), median (quartiles)	84.5 (63.8–95.3)	107.5 (96.3–131.8)	0.015*	NA	NA
Time until renal vein exposure (min), median (quartiles)	21.0 (16.5–27.0)	37.0 (28.3–55.0)	0.001*	NA	NA
Time between renal vein exposure and ligation (min), median (quartiles)	6.5 (3.0–13.5)	16.5 (13.0–32.3)	0.002*	NA	NA
Days of hospital stay (days), median (quartiles)	2.5 (2.0–3.3)	3.0 (2.0–4.3)	0.405	NA	NA
Complications, n (%)	3 (18.8%)	3 (18.8%)	1	0	1
Clavien-Dindo classification, n (%)			1		1
I	0	1 (6.3%)		0	
II	2 (12.5%)	1 (6.3%)		0	
IVa	1 (6.3%)	1 (6.3%)		0	
Pathological parameters:					
Tumor maximum diameter (mm), median (quartiles)	50.0 (40.0–107.5)	64.5 (45.0–70.5)	0.890	NA	NA
Histologic type, n (%)			0.281		1
Clear cell	6	11		0	
Papillary type 1	0	1		0	
Papillary type 2	1	1		0	
Cromophobe	3	0		0	
Other RCC	2	2		0	
Oncocytoma	3	1		0	
Xantogranulomatous Pyelonephritis	1	0		0	
Pathology grade (Fuhrman), n (%)			0.793		1
1	3	2		0	
2	8	11		0	
3	0	0		0	
4	0	1		0	
Microvascular invasion, n (%)	2	3	1	0	1
Lymphatic invasion, n (%)	1	1	1	0	1

	NT group n = 16	C group n = 16	$p^{\dagger}$	Control group n = 9	$p^{\ddagger}$
Renal vein (segmental) invasion, n (%)	0	4	0.106	0	1
Collecting system invasion, n (%)	1	1	1	0	1
Perirenal fat invasion, n (%)	3	3	1	0	1
pT, n (%)			0.208		1
pT1a	3	4		0	
pT1b	5	3		0	
pT2a	0	3		0	
pT2b	2	0		0	
pT3a	2	5		0	
pT3b	0	0		0	
pN, n (%)			NA		NA
pN0	4	6		0	
pN1	0	0		0	
Positive surgical margins, n (%)	0	0		NA	

* clinically significant; † comparison between no-touch and control groups; ‡ comparison between intervention and control groups. BMI, body mass index; C, control group; CKD, chronic kidney disease; ECOG, Eastern Cooperative Oncology Group performance status; GFR, glomerular filtration rate; NT, no-touch; M, male; min, minute; mL, milliliter; n, number; NA, not available; RCC, renal cell carcinoma.

Groups were balanced regarding all demographic and clinicopathological characteristics. The operative time was significantly shorter in the NT group compared to the C group (84.5 minutes [min], interquartile range [IQR] 31.5 vs. 107.5 min, IQR 35.5; p = 0.015), as was the time to renal vein exposure (21.0 min, IQR 10.5 vs. 37.0 min, IQR 26.7 minutes; p < 0.001).

Five patients had non-malignant histology: oncocytoma in four and focal xanthogranulomatous pyelonephritis (XP) in one. Only RCC patients were included for CTC and CMC cell counts in the intervention groups.

Baseline (S0) CC counts in intervention and control groups are shown in Table 2 and Fig. 2.

Table 2
Baseline (S0) circulating cell counts and characterization in the intervention and control groups

	Control group n = 9		Intervention group (S0) n = 31									
	HC n = 5	IC n = 4	RCC NT n = 12	RCC C n = 15	Oncocytoma n = 4	IC vs. HC *p* value	IC vs. RCC C *p* value	IC vs. RCC NT *p* value	IC vs. O *p* value	O vs. HC *p* value	O vs. RCC C *p* value	O vs. RCC NT *p* value
Single CTCs	0	0	0	0.33 (0,0,5)	0	1	1	1	1	1	1	1
Epithelial CTCs	0	0	0	0.33 (0,0,5)	0	1	1	1	1	1	1	1
EMT CTCs	0	0	0	0	0	NA	NA	NA	NA	NA	NA	NA
CMCs	0	22.5 (1,18.5,52)	3.0 (0,1.0,12)	8.47 (0,4.0,33)	24.75 (8,17.0,57)	0.017*	0.874	0.233	1	0.002*	0.205	0.037*
Single CMCs	0	11.75 (1,12.0,22)	2.58 (0,1.0,11)	7.2 (0,4.0,31)	14.25 (8,15.0,19)	0.018*	1	0.227	1	0.003*	0.371	0.052
CMCs in clusters	0	10.75 (0,6.5,30)	0.42 (0,0,3)	1.27 (0,0,10)	10.5 (0,2.0,38)	0.174	0.431	0.314	1	0.213	0.567	0.387
Clusters	0	2 (0,1.0,6)	0.17 (0,0,1)	0.4 (0,0,3)	2 (0,1.0,6)	0.202	0.520	0.360	1	0.202	0.520	0.360
Total CCs	0	22.5 (1,18.5,52)	3.0 (0,1.0,12)	8.8 (0,5.0,33)	24.75 (8,17.0,57)	0.017*	1	0.234	1	0.002*	0.269	0.030*
Single CCs	0	11.75 (1,12.0,22)	2.58 (0,1.0,11)	7.53 (0,5.0,31)	14.25 (8,15.0,19)	0.019*	1	0.225	1	0.003*	0.476	0.042*

Values are presented as means (minimun, median, maximum); * clinically significant; p-values calculated using the Kruskal-Wallis test for each cell count variable to assess the homogeneity of the four groups, followed by pairwise comparisons (using Dunn's test) with Bonferroni correction. C, conventional; CC, circulating cell; CMC, circulating mesenchymal cell; CTC, circulating tumor cell; EMT, epithelial-to-mesenchymal transition; HC, healthy control; IC, inflammatory control; n, number; NA - not available; NT, no-touch; RCC, renal cell carcinoma; O - oncocytoma; S0, OR arrival time-point.

No CTCs were detected in the control groups and in oncocytoma patients. No CMCs were detected in the healthy control group (Fig. 2). However, all patients in the chronic inflammation control group had a significant number of CMCs with a mean of 22.5 (range 1–52) cells per 7.5 mL of blood (p = 0.007 compared to healthy controls), with no difference to cancer patients in the intervention groups (p = 0.291 and 0.460, for NT and C groups, respectively). CMCs were also detected in all oncocytoma patients, with no difference to the RCC C group (p = 1.000) or chronic inflammation patients (p = 0.205), but with higher counts compared to the RCC NT group (p = 0.037).

No correlation was found between CMC counts and serum inflammatory parameters, namely C-reactive protein, leucocyte, and neutrophil counts.

Table 3 shows the CC counts and characteristics in each intervention group at each considered time point.

Table 3
CC counts and characterization in each study group and time point.

	RCC NT group (n = 12)				RCC C group (n = 15)			
	S0	S1	D1	D30	S0	S1	D1	D30
Single CTCs	0	0	1.36 (0,0,13)	0.09 (0,0,1)	0.33 (0,0,5)	0.07 (0,0,1)	0.07 (0,0,1)	0.36 (0,0,4)
Epithelial CTCs	0	0	0.36 (0,0,2)	0.09 (0,0,1)	0.33 (0,0,5)	0.07 (0,0,1)	0.07 (0,0,1)	0.36 (0,0,4)
EMT CTCs	0	0	1.00 (0 0,11)	0	0	0	0	0
CMCs	3.00 (0,1.00,5)	2.00 (0,0,11)	1.91 (0,0,12)	0.91 (0, 0, 7)	8.47 (0,4.00,33)	7.00 (0,4.00,31)	1.43 (0,1.00,6)	1.57 (0,0.50,11)
Single CMCs	2.58 (0,1.00,11)	2.00 (0,0,11)	1.91 (0,0,12)	0.45 (0, 0, 2)	7.20 (0,4.00,31)	6.27 (0,4.00,22)	1.43 (0,1.00.6)	1.21 (0,0.50,6)
CMCs in clusters	0.42 (0,0,3)	0	0	0.45 (0, 0, 5)	1.27 (0,0,10)	0.73 (0,0,11)	0	0.36 (0,0,5)
Clusters	0.17 (0,0,1)	0	0	0.18 (0, 0, 2)	0.40 (0,0,5)	0.20 (0,0,3)	0	0.07 (0,0,1)
Total CCs	3.00 (0,1.00,12)	2.00 (0,0,11)	7.00 (0,1.00,25)	1.00 (0, 0, 8)	8.80 (0,5.00,33)	7.07 (0,4.00,31)	1.5 (0,1.00,6)	1.93 (0,1.00,11)
Single CCs	2.58 (0,1.00,11)	2.00 (0,0,11)	3.27 (0,1.00,25)	0.55 (0, 0, 2)	7.53 (0,5.00,31)	6.33 (0,4.00,22)	1.5 (0,1.00,6)	1.57 (0,1.00,6)

	Oncocytoma (n = 4)				Xanthogranulomatous Pyelonephritis (n = 1)			
	S0	S1	D1	D30	S0	S1	D1	D30
Single CTCs	0	0	0	0	0	0	0	0
Epithelial CTCs	0	0	0	0	0	0	0	0
EMT CTCs	0	0	0	0	0	0	0	0
CMCs	24.75 (8,17.00,57)	13.5 (1,11.00,31)	0.75 (0,0.50,2)	5.50 (0 6.00,12)	4	0	1	1
Single CMCs	14.25 (8,15.00,19)	6.25 (1,6.00,12)	0.75 (0,0.50,2)	4.75 (0,5.00,9)	4	0	1	1
CMCs in clusters	10.50 (0,2.00,38)	7.25 (0,1.00,27)	0	0.75 (0,0,3)	0	0	0	0
Clusters	2.00 (0,1.00,6)	0.50 (0,0.5,1)	0	0.25 (0,0,1)	0	0	0	0
Total CCs	24.75 (8,17.00,57)	13.50 (1,11.00,31)	0.75 (0,0.50,2)	5.50 (0,6.00,10)	4	0	1	1
Single CCs	14.25 (8,15.00,19)	6.25 (1,6.00,12)	0.75 (0,0.50,2)	4.75 (0,5.00,9)	4	0	1	1

Values presented as means (minimum, median, maximum); S0, OR arrival time-point; S1, specimen extraction time-point; D1, postoperative day 1 time-point; D30, postoperative day 30 time-point; C, conventional; CC, circulating cells; CMCs, circulating mesenchymal cells; CTCs, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; NT, no-touch; RCC, renal cell carcinoma.

The total CC detection rates in the NT, C, and overall RCC groups were 58.3%, 80.0%, and 70.4% at S0, 41.6%, 86.7%, and 66.7% at S1, 50.0%, 64.3%, and 60.0% at D1, and 54.5%, 42.9%, and 44.0% at D30, respectively.

The CTC detection rates in the NT, C, and whole RCC groups were 0%, 6.7%, and 3.7%, on both S0 and S1, 27.3%, 7.1%, and 16.0% on D1, and 9.1%, 14.3%, and 12.0% on D30, respectively.

Most CCs were CMCs in all groups and time points. Single CMCs were found in all time points and every group, in one patient with focal XP at S1. CMC clusters were found in every group at all time points except at D1, and in the NT and XP groups at S1.

The CMC detection rate in the RCC NT, RCC C, and whole RCC groups was 58.3%,73.3%, and 81.5% at S0, 41.7%, 80.0%, and 63.0% at S1, 36.4%, 57.1%, and 48.0% at D1, and 45.5%, 42.9%, and 44.0% D30, respectively.

Clusters of CMCs were found in 16.7%, 20.0%, and 18.5% of patients in the NT, C, and whole RCC groups at S0, 0%, 6.7%, and 3.7% at S1, 0%, 0%, and 0% at D1, and 13.3%, 0.0%, and 7.4% at D30.

A progressive decline in total CCs and CMCs after surgery was observed in both intervention groups, but it was only significant in the C group (Fig. 3). This decrease was mainly due to the significant decline in CMCs at D1 (from 4.78 to 1.64 CMCs/7.5 mL of blood; p = 0.035) since no differences were found between S0 and S1 nor between D1 and D30. In all intervention groups, CMC clusters disappeared at D1 and reappeared at D30.

Table 4 shows CC counts and variation between intervention groups and time points.

Table 4
CTC counts and variation in each intervention group and time point (primary outcome)

	S0			S1			D1			D30			Cell count difference S1 - S0			Relative cell coun difference S1 - S0 (%)	
	NT	C	*p**	NT	C	*p**	NT	C	*p**	NT	C	*p**	NT	C	*p*‡	NT	C
Single CTCs	0	0.33	0.371	0	0.07	0.371	1.36	0.07	0.169	0.09	0.36	0.662	0	-0.26	0.412	NA	-80
Epithelial CTCs	0	0.33	0.371	0	0.07	0.371	0.36	0.07	0.169	0.09	0.36	0.662	0	-0.26	0.412	NA	-80
EMT CTCs	0	0	NA	0	0	NA	1	0	0.259	0	0	NA	0	0	NA	NA	NA
CMCs	3.00	8.47	0.251	2.00	7.00	**0.033**	1.91	1.43	0.554	0.91	1.57	0.302	-1.00	-1.47	0.883	-33.3	-17.3
Single CMCs	2.58	7.20	0.203	2.00	6.27	**0.033**	1.91	1.43	0.554	0.45	1.21	0.235	-0.58	-0.93	0.677	-22.6	-13.0
CMCs in clusters	0.42	1.27	0.746	0.00	0.73	0.371	0.00	0.00	NA	0.45	0.357	0.861	-0.42	-0.54	1	-100.0	-42.1
Clusters	0.17	0.40	0.719	0.00	0.20	0.371	0.00	0.00	NA	0.18	0.07	0.846	-0.17	-0.20	1	-100.0	-50.0
Total CCs	3.00	8.80	0.142	2.00	7.07	0.021	7.00	1.50	0.775	1.00	1.93	0.373	-1.00	-1.73	0.961	-33.3	-19.7
Single CCs	2.58	7.53	0.106	2.00	6.33	0.021	3.27	1.50	0.775	0.55	1.57	0.270	-0.58	-1.20	0.826	-22.6	-15.9

Values presented as means; * Kruskal-Wallis test; ‡ Wilcoxon test.

S0, blood sample collected at OR arrival; S1, blood sample collected at specimen extraction; D1, blood sample collected at postoperative day 1; D30, blood sample collected at postoperative day 30; NT, no-touch group; C, conventional group; CC, circulating cell; EMT, epithelial-to-mesenchymal transition; CMC, circulating mesenchymal cell; CTC, circulating tumor cell; NA, impossible to determine.

No significant differences were found between intervention groups in absolute and relative CTC and CMC variations between S0 and the remaining time points, suggesting that there is no reduction in CTC or CMC release with NT RN. However, a significantly lower CMC count was found in the NT arm compared to the C arm at S1 (4.38 vs. 7.06, p = 0.044). There were no differences in CC counts at any other time point.

In the whole RCC cohort, moderate negative correlations were found between CT tumor contrast washout and CMCs at S1 (r=-0.503, p = 0.008, and r=-0.425, p = 0.027, for corticomedullary and late phases respectively), and CMC clusters at S0 (r=-0.526, p = 0.005, and r=-0.442, p = 0.021, for corticomedullary and late phases, respectively). Thus, the greater the washout, the fewer CMCs (Supplementary Material).

Total CC counts in S0 and ∂S1-S0 correlated with MAP score[24] (p = 0.031 and 0.020, respectively). The same was true for CMC counts at ∂S1-S0 (P = 0.024).

No differences were found in CC counts according to TNM stage, histologic type (clear vs. non-clear cell), ISUP pathologic grade, PADUA score[25], R.E.N.A.L. score[26], and tumor size (Supplementary Material). Additionally, no significant correlation was found between CTC or CMC counts and tumor diameter, volume, parenchymal contact area, or attenuation value measured by CT scan.

At a median follow-up of 12.5 months, no difference was found in complication rates, PFS, or OS between groups (Supplementary Material). Subgroup analysis by CC type and count showed no survival difference either.

3. DISCUSSION

This study suggests that a NT LRN does not reduce CTC or CMC release or impact survival. It also shows no increase in CC release with surgical manipulation. Similar findings by Haga *et al.* showed no difference in CTC counts after LRN, contrary to open RN (7.7 ± 6.8 to 22.5 ± 26.3, p < 0.05)[27]. The study suggested that minimally invasive surgery reduced CC release. Conversely, two studies reported an increased CTC detection rate after RCC surgery but CTC counts were not studied[12,14].

In the present study, a progressive reduction in CMC counts was observed over time, most of which at D1. This was significant only in the C group, probably due to a random higher baseline count and small sample size. Similar findings of a progressive decrease in CTC counts during follow-up in M0 patients were reported by Wang *et al*[28].

CTC detection rates were low, possibly due to the predominance of localized low-stage tumors. Additionally, RCC typically has lower epithelial CTC counts due to a higher incidence of EMT compared to other tumor types, with loss of epithelial markers[5,6]. Although a progressive CTC decline was observed across time points, it was not significant, probably due to low baseline counts and small sample size.

The only clinicopathological parameter that correlated with total CC and CMC counts was MAP score, an imagiological surrogate marker of peri-renal inflammation. No other clinicopathologic or laboratory parameters correlated with CC counts and the same was observed for tumor diameter, volume, parenchymal contact area, or attenuation value.

The NT technique proved to be faster and as safe as the C technique. NT RN took on average 23 minutes less than the C technique, which is an advantage *per se* and may favor the choice of this technique.

The healthy control group confirmed no CCs. However, despite the absence of CTCs, a significant number of CMCs were identified in all patients of the chronic inflammation control group. The same was true for oncocytoma patients. None of these patients developed cancer during follow-up. Furthermore, CMC counts in these two groups did not differ from RCC patients. This was surprising because the literature reports the absence of CCs in controls using the same criteria and raises the question of the nature of CMCs[5]. The scientific community has been naming these cells as mesenchymal CTCs. To our knowledge, no study to date has included an inflammation control group. We may wonder if these cells are CTCs or a subset of leukocytes with low CD45 expression, cancer-associated fibroblasts, or another inflammatory cell. They may even be different cell types. There is a well-studied relationship between cancer and inflammation that may help explain these findings[29]. The correlation between CMC counts and MAP scores further points in this direction. Nevertheless, the decrease in CMC counts after surgery suggests that they are mainly from tumor origin. Characterization of CCs with four markers is a limitation of most detection devices[5]. Caution should be exercised in classifying these cells as CTCs. Future downstream CMC analysis and improved biomarker selection are warranted to elucidate their true nature.

The main limitations of this study were its small sample size, low power to detect small differences in CC counts between groups and time points, and the short follow-up, hindering clinical outcome analysis. Additionally, CC downstream analysis was not performed.

Peri-operative CTC kinetics is still poorly understood, and its clinical significance remains unclear. This study suggests no advantage in early pedicle ligation. Has the last Robson principle fallen? Larger studies are crucial to confirm this hypothesis. Furthermore, a more comprehensive analysis of CCs and their interaction with the immune system may increase the understanding of RCC and improve future treatment protocols.

4. MATERIALS (PATIENTS) AND METHODS

4.1. Study design and participants

A prospective randomized controlled trial was conducted at the Department of Urology of Centro Hospitalar Universitário Lisboa Norte (CHLUN) between September 2021 and April 2022 (Supplementary Figure S1). Patients presenting with a renal mass and indication for LRN were enrolled and randomized on a 1:1 ratio to a NT LRN (NT group) or a C LRN (C group). Exclusion criteria were patients with a history of another cancer, < 18 years old, pregnant women, and surgically unfit patients. All patients gave written informed consent.

Patients were positioned in lateral decubitus. Two 12mm ports were inserted, on the para-rectal and midclavicular lines, and two 5mm ports were placed, one in the midaxillary line, and the fourth 2cm away from the anterior superior iliac spine. An optional 5mm port was placed for liver retraction. The colon was retracted along the avascular plane just anterior to the Gerota's fascia until the vena cava on the right side and the aorta on the left side were exposed. The second part of the duodenum was reflected medially on the right side.

Hereafter, the surgical protocol for the NT group was to incise the Gerota's fascia just above the renal vein and to immediately and selectively ligate the renal pedicle using Hem-o-Lok® clips. No kidney manipulation was done until this point. The surgery then proceeded in the usual way.

In the C group, the surgical protocol entailed the identification and superior retraction of the ureter together with the kidney's lower pole, while the dissection continued cephalad until the renal pedicle was reached. The latter was then selectively ligated as previously described while maintaining renal traction.

Patients presenting for a total nephrectomy due to hypo-functioning kidneys and no history of cancer were used as controls and divided into two subgroups: 1) patients with systemic inflammation (inflammatory controls), defined by severe/recurrent pyelonephritis/pyonephrosis, elevated serum inflammatory parameters, and chronic pyelonephritis on pathology; and 2) patients with atrophic kidneys and none of the above criteria (healthy controls).

Study approved by the Centro Hospitalar Universitário Lisboa Norte (CHULN) Ethics Committee, and all methods were carried out with conforming to CONSORT and the Declaration of Helsinki. Clinicaltrials.gov: NCT05070637, 07/10/2021.

4.2. Randomization and masking

Patients were randomly assigned (simple 1:1 randomization) to either group using a computer-generated allocation sequence. The allocation was disclosed to the surgical team upon patient's arrival at the operating room (OR), with sequentially numbered envelopes.

4.3. Blood sample collection, CTC isolation, and characterization

A 7.5 mL peripheral venous blood sample was collected in EDTA tubes upon arrival to the OR (S0), after specimen extraction (S1), and at postoperative day one (D1), and 30 (D30). A single blood sample was collected from study controls on the day of enrollment.

Whole blood samples were anonymized, coded, and injected into the RUBYchip™ microfluidic device at 80 µL/min, as described elsewhere[30]. The characterization of CCs was performed by immunocytochemistry using AF647-conjugated anti-human vimentin (Vim; Biolegend, 1:50), PE-conjugated anti-

human CD45 (Invitrogen, Thermo Fisher Scientific, 1:50), FITC-conjugated anti-human cytokeratin (CK), and DAPI (1 µg/mL). Fluorescent images were captured with an Allegro Plus (BioView, Israel) microscope at 20x magnification.

CCs were identified and characterized by morphological criteria (brightfield cell membrane integrity, round nucleus, cell-like morphology) and phenotypical criteria (DAPI+/CD45-/CK + for epithelial CTCs, and DAPI+/CD45-/CK+/Vim + for epithelial-mesenchymal transition CTCs). Circulating mesenchymal cells (CMCs) were identified as DAPI+/CD45-/CK-/Vim+. Groups of at least two cells with the above features were considered cell clusters.

4.4. Outcomes and statistical analysis

A sample size of 34 patients was calculated to detect a 20% decrease in CTCn variation after surgery in the NT group, assuming a Poisson distribution. The Kruskal-Wallis test was used to assess group homogeneity, followed by pairwise comparisons (Dunn's test). To compare CC counts between groups and time points, the Wilcoxon signed-rank test was used. The Mann-Whitney test was applied to compare absolute cell count differences between groups. The Wilcoxon rank-sum test was used to compare relative cell count differences between groups. Spearman's correlation analysis was done between cell counts and quantitative clinical and imaging variables. Kaplan-Meier was used for progression-free (PFS), and overall survival (OS) analyses. Bonferroni corrections were used for multiple hypotheses testing. A significance level of 0.05 was considered. Statistical analyses were performed with R Software v2022.07.1 (R Foundation for Statistical Computing, Vienna, Austria).

5. CONCLUSIONS

NT LRN did not reduce CC release or improve survival compared with C LRN. However, it proved to be faster and as safe as the conventional technique. CMCs were found in chronic inflammation and oncocytoma patients and decreased after surgery, suggesting tumor origin but questioning their CTC status.

Declarations

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AUTHOR CONTRIBUTIONS:

Tito Palmela Leitão had full access to all study data and took responsibility for its integrity and accurate analysis.

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Drafting of the manuscript: Tito Palmela Leitão, Patrícia Corredeira.

Critical revision of the manuscript for important intellectual content: Paulina Piairo, Carolina Rodrigues, Ana Cavaco, José Palma Reis, Tomé Lopes, Lorena Diéguez, Luís Costa.

Statistical analysis: Tito Palmela Leitão, Patrícia Corredeira, Marília Antunes.

Obtaining funding: Tito Palmela Leitão, Luís Costa, Lorena Diéguez.

Administrative, technical, or material support: Patrícia Corredeira, Sandra Kucharczac, Miguel Miranda.

Supervision: Luis Costa, Lorena Diéguez, Tomé Lopes.

Other (specify): None.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS STATEMENT

I certify that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript, are the following:

- LD and PP are co-founders and managers of RUBYnanomed.
- TPL is owner of one of the funding parties: Palmela Leitão Urologia Lda.
- PC, CR, MM, AC, SK, MA, SP, JPR, TPL and LC have no conflict of interests to declare.

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PATENTS

The RUBYchip™ is based on patent PCT/EP2016/078406, filed by INL in front of EPO on 22 November 2016, covering the geometry and surface coating of the microfluidic system for CTC isolation, and currently licensed exclusively to RUBYnanomed.

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Figures

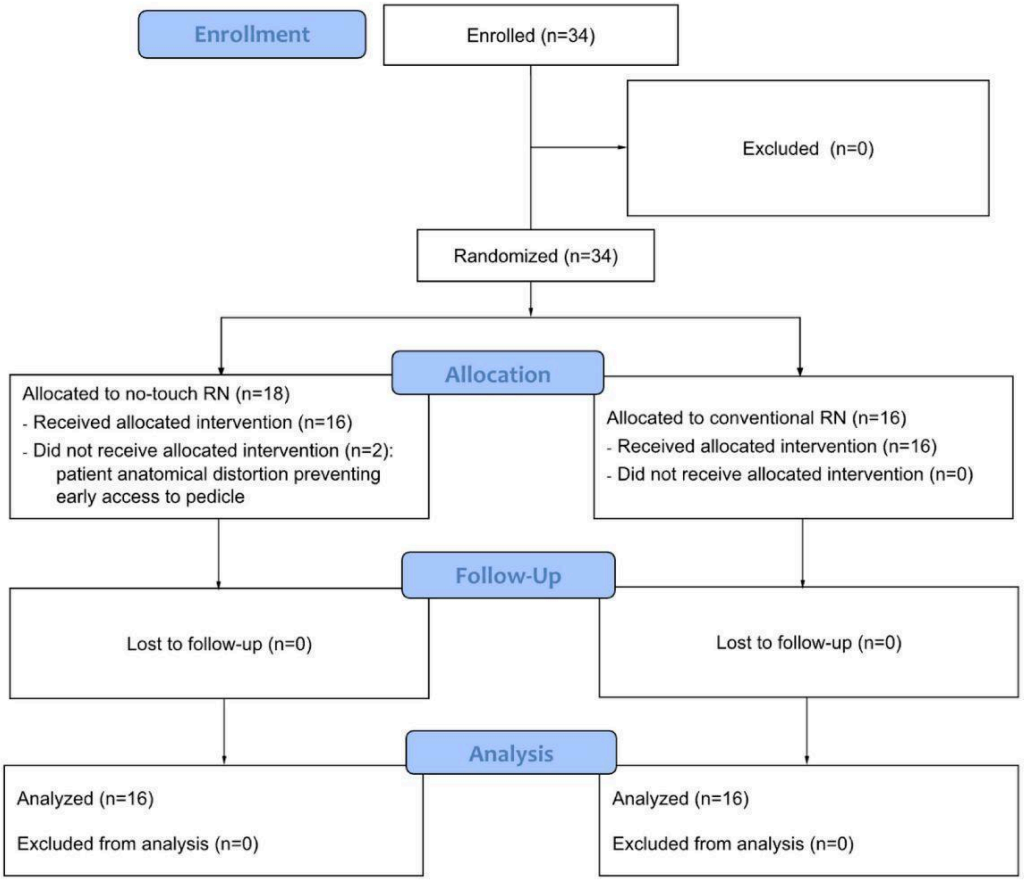

Figure 1

CONSORT flow diagram

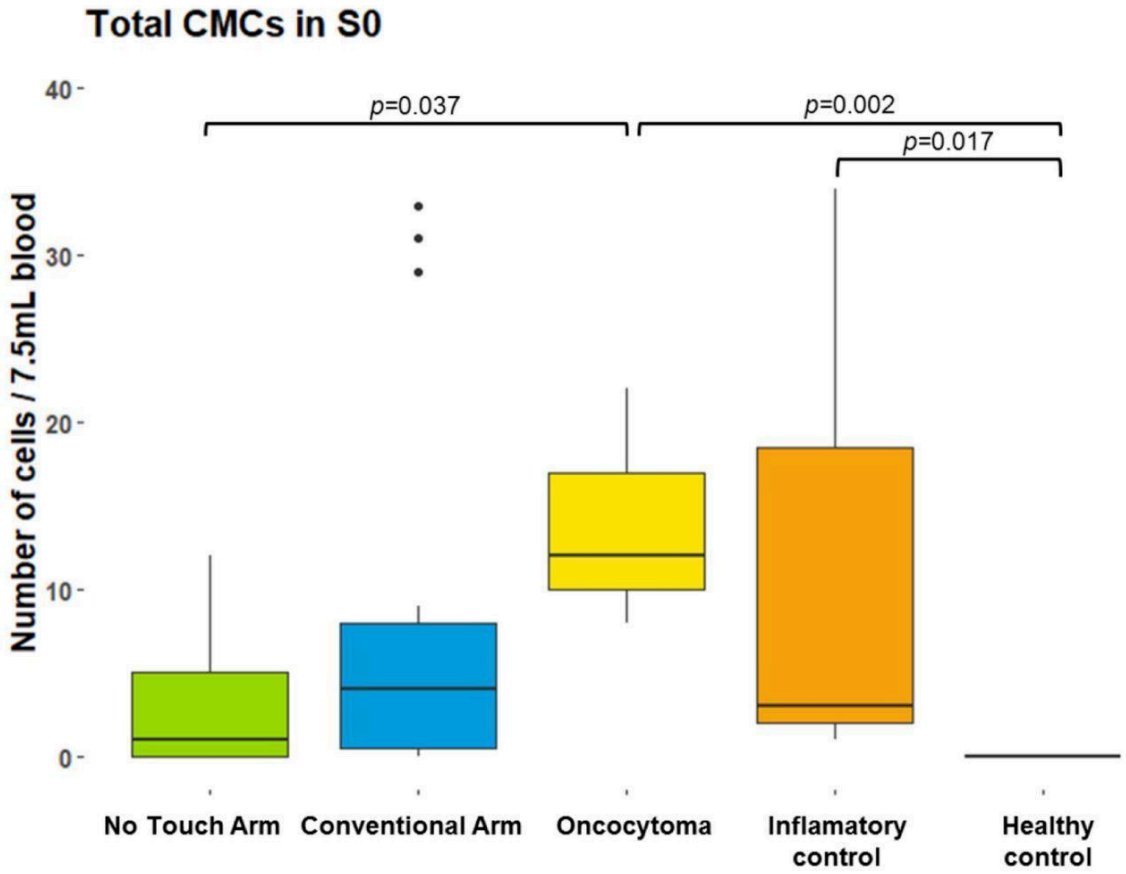

Figure 2

Box-plots comparing total CMC counts at S0 between NT group, C group, oncocytoma, inflammatory controls, and healthy controls.

CMC, circulating mesenchymal cells; NT, no-touch; S0, baseline (arrival at the operating room).

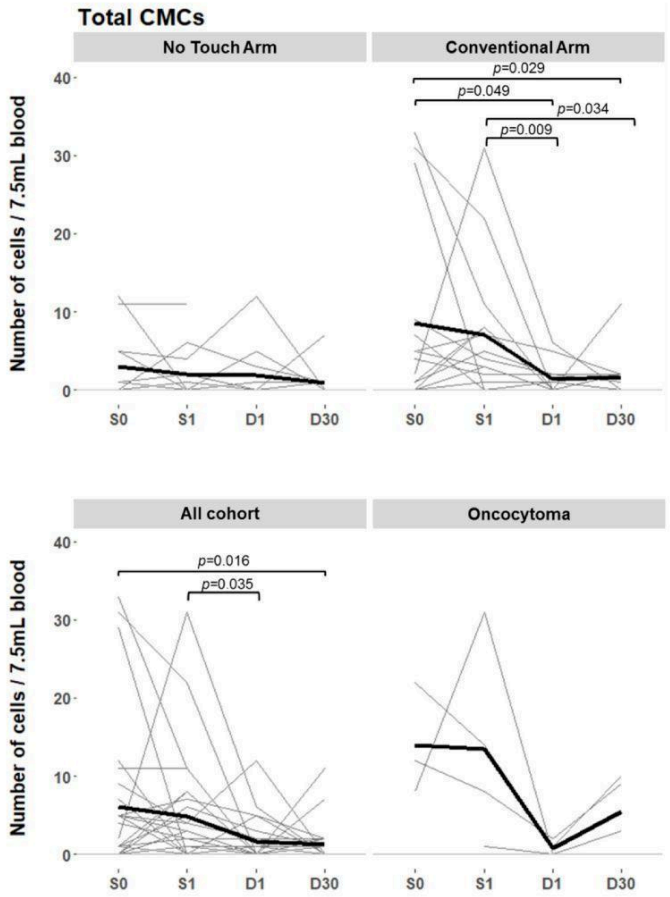

Figure 3

CMC counts at different time points (S0, S1, D1, and D30) for the NT and C groups (top figure), and for the entire cohort and oncocytoma groups (bottom figure). Individual patients are represented as grey lines and the mean is represented as a black line.

S0 - blood sample collected at OR arrival; S1 - blood sample collected at specimen extraction; D1 - blood sample collected at postoperative day 1; D30 - blood sample collected at postoperative day 30; NT - no-touch group; C - conventional group; CMCs - circulating mesenchymal cells.

Supplementary Files

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