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The Prognostic Utility of *KRAS* Mutations in Tissue and Circulating Tumour DNA in Colorectal Cancer Patients

Joel Petit [1,2,3,*], Georgia Carroll [1,3,4], Jie Zhao [1], Peter Pockney [2,3] and Rodney J. Scott [2,3,5]

[1] Division of Surgery, John Hunter Hospital, New Lambton Heights, NSW 2305, Australia
[2] School of Biomedical Sciences and Pharmacy, University of Newcastle, New Lambton Heights, NSW 2305, Australia
[3] Hunter Medical Research Institute, University of Newcastle, New Lambton Heights, NSW 2305, Australia
[4] School of Medicine and Public Health, University of Newcastle, New Lambton Heights, NSW 2305, Australia
[5] NSW Health Pathology, New Lambton Heights, NSW 2305, Australia
* Correspondence: c3286347@uon.edu.au

Abstract: This study aims to investigate the long-term prognostic utility of circulating tumour DNA (ctDNA) *KRAS* mutations in colorectal cancer (CRC) patients and compare this with *KRAS* mutations in matched tissue samples. Tumour tissue (n = 107) and ctDNA (n = 80) were obtained from patients undergoing CRC resection and were analysed for *KRAS* mutations. The associations between *KRAS* mutation and overall survival (OS), cancer-specific survival (CSS), and recurrence-free survival (RFS) were analysed. All outcomes were measured in years (y). A total of 28.8% of patients had *KRAS* mutations in ctDNA and 72.9% in tumour tissue DNA. The high frequency of *KRAS* mutations in tissue samples was due to 51.4% of these being a detectable low mutation allele frequency (<10% MAF). Comparing *KRAS* mutant (*KRAS*mut) to *KRAS* wild-type (*KRAS*wt) in ctDNA, there was no association found with OS (mean 4.67 y vs. 4.34 y, p = 0.832), CSS (mean 4.72 y vs. 4.49 y, p = 0.747), or RFS (mean 3.89 y vs. 4.26 y, p = 0.616). Similarly, comparing *KRAS*mut to *KRAS*wt in tissue DNA there was no association found with OS (mean 4.23 y vs. 4.61 y, p = 0.193), CSS (mean 4.41 y vs. 4.71 y, p = 0.312), or RFS (mean 4.16 y vs. 4.41 y, p = 0.443). There was no significant association found between *KRAS* mutations in either tissue or ctDNA and OS, CSS, or RFS.

Keywords: colorectal cancer; prognosis; tumour biomarkers; circulating cell-free DNA; circulating tumour-DNA; *KRAS*

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1. Introduction

Colorectal cancer (CRC) was responsible for an estimated 862,000 deaths globally in 2018 which makes it the second leading cause of cancer-related death [1]. Advances in treatment and early detection strategies over the last decade have significantly changed the medical management of this disease and improved overall survival. However, the outcomes for CRC patients remain closely related to the stage of cancer at diagnosis. The 5 year survival rate of patients is inversely related to the stage of the disease. At stage I, there is a 99% survival rate, at stage II it is 89%, at stage III it is 71%, and at stage IV it drops precipitously to 13% [2]. Whilst most patients with stage II or III disease have good outcomes, there is a proportion of patients who are affected by disease recurrence. It has been shown that there is a survival benefit to offering adjuvant chemotherapy to patients with stage III disease; however, this benefit has not been seen in patients with stage II disease [3]. Despite the absence of benefit in patients with stage II disease, there may be a subgroup who would benefit if they were able to be identified by appropriate biomarkers after surgical resection with curative intent. To date, it has not been possible to identify which patients with stage II or III disease will develop recurrence. However, recent evidence from Tie et al., who used sequencing to identify multiple mutations from primary tumour tissue and plasma ctDNA that subsequently guides therapeutic choices

in stage II colon cancer patients, has shown non-inferiority to standard management [4]. By using a ctDNA-guided approach there was a reduced use of adjuvant chemotherapy without a significant compromise in recurrence-free survival (RFS).

The potential of using *KRAS* mutations in primary tumour samples as a prognostic or predictive marker has been proposed by many researchers. Early studies suggested that *KRAS* mutations could be used as a prognostic marker; however, there have been conflicting results from more recent studies [5–30]. Almost half the research in this field has found no significant association between *KRAS* mutation status and prognostic outcomes of OS, CSS, or RFS. During the last few years, the research in this area has become even more complicated since more recent studies have replaced tissue tumour samples with ctDNA analysis and the concordance of mutational status between tumour tissue samples and ctDNA varies widely between studies. Most of these studies on tissue and ctDNA have been small, with around 100 patients. *KRAS* mutations found in these different types of samples could have diverse prognostic or predictive utility and caution should be used when comparing results from these studies. This study aims to add to the evidence regarding possible utility of the long-term prognostic ability of ctDNA *KRAS* mutation detection in colorectal cancer patients and compare this with *KRAS* mutations in matched tissue samples.

2. Materials and Methods

2.1. Sample Collection and Ethics

Specimens were collected from consecutive patients undergoing a CRC resection between July 2011 and December 2013 at either the John Hunter Hospital or Newcastle Private Hospital. The tissue samples were collected from the primary tumour at the time of resection prior to fixation and snap frozen and stored at −80 °C. The blood samples were collected in either K2-EDTA tubes or lithium heparin tubes pre-operatively and were processed within 4 h of phlebotomy. Two centrifugation steps were used to prepare plasma samples, which were then stored at −80 °C (Supplementary S1). For the purposes of this study, a total of 121 patients had suitable specimens that were able to be utilised for either plasma (n = 80) or tissue (n = 107) analysis. A total of 66 patients had both tissue and plasma available for analysis. Histopathological examination and status of the tumour was confirmed by a certified pathologist and staged using the TNM system defined by the Union for International Cancer Control (UICC) [31]. Collected patient characteristics included age, gender, body mass index (BMI), smoking status, comorbidities graded as the Charlson Co-morbidity Index (CCI), site of tumour, histopathology, tumour staging, use of adjuvant therapy, disease recurrence, and mortality. This study was conducted in accordance with the Helsinki Declaration and was approved by the Hunter New England Human Research and Ethics Committee (2019/ETH01147, 2019/ETH10205). Patient consent for specimen collection and analysis was obtained prior to their procedures in all cases.

2.2. DNA Extraction

A standard ethanol and salt extraction method was used to isolate genomic DNA from the fresh frozen tissue samples (Supplementary S1). The genomic tissue DNA was then prepared for droplet digital polymerase chain reaction (ddPCR) using Zymo DNA Clean and Concentrator kits (Zymo Research, Irvine, CA, USA). Purification of DNA from plasma was performed using Zymo Quick-cfDNA Serum and Plasma kits. The total amount of genomic DNA purified from the plasma samples was quantified using Qubit 2.0, dsDNA high-sensitivity assay (Life Technologies, Carlsbad, CA, USA). All methods were performed according to the manufacturer's instructions.

2.3. KRAS-Mutation Testing Using ddPCR

Genomic DNA extracted from plasma and tissue were analysed for 7 *KRAS* mutations (G12A, G12C, G12D, G12R, G12S, G12V, and G13D) by ddPCR using the *KRAS* G12/G13 Screening kit (Bio-rad, Hercules, CA, USA). *KRAS* multiplex analysis was performed using

1–8 µL volume of sample DNA. *KRAS* master mixes were made for all different volumes used in each run. For each reaction, the master mix contained 1.1 µL of *KRAS* primer/probe mix, 11 µL of ddPCR Supermix (Bio-rad, Hercules, CA, USA), and autoclaved Millipore water in variable volumes relative to the sample input volume. The sample and master mix were combined to achieve a total end volume of each PCR reaction of 22 µL. The 96-well plate was then sealed, centrifuged at 300 rpm for 5 s, gently vortexed, and recentrifuged at 300 rpm. The plate seal was removed, and the plate was then run on the QX200 AutoDG Droplet Digital PCR system, immediately foil heat sealed using the PX1 PCR Plate Sealer, and run on the C1000 Touch Thermocycler. The PCR cycling conditions were as per the manufacturer's instructions. The plate was then placed into the QX200 Droplet Reader for analysis, and the data were analysed using QuantaSoft software v1.2 (Bio-rad, Hercules, CA, USA). For each PCR plate, there were control samples for both mutation and wild-type *KRAS* that were made using Horizon reference standards. All assays included a no template control (NTC).

2.4. Calculation of the LoD and LoB

The limit of detection (*LoD*) and limit of blank (*LoB*) for the ddPCR analysis were measured according to the methods described by Armbruster et al. [32]. The *LoB* was determined by 22 replicates of PCR wells that contained only *KRAS* wild-type controls. The *LoD* was determined by the following equation:

$$LoD = LoB + 1.645(SDblank)$$

The *LoD* using this method was 4.73 droplets. The cut-off for a positive *KRAS* mutation call was, therefore, conservatively placed at 6 or more positive droplets. Alternate cut-offs of more than 1%, 5%, and 10% mutation allele frequency (MAF) were also used for the tissue cohort in separate analyses.

2.5. Statistical Analysis

Overall survival (OS) was defined as the time from the cancer operation until death from any cause. Cancer-specific survival (CSS) was defined as the time from the cancer operation until death from colorectal cancer. Patients were excluded from OS and CSS analysis if they had metastatic disease that did not undergo an R0 resection plus metastasectomy. Recurrence-free survival (RFS) was defined as the time from the cancer operation until the first objective evidence of disease progression or death from colorectal cancer. Patients were excluded from RFS analysis if they survived < 30 days from the initial operation or had metastatic disease that did not undergo an R0 resection plus metastasectomy. The survival and recurrence of patients in each *KRAS* mutation group were examined using Kaplan–Meier curves and stratified by two potential prognostic factors: *KRAS* mutation in tissue and cell-free *KRAS* DNA. The analysis was performed using both total available survival data for each individual as well as survival data adjusted to an endpoint of 5 years.

Associations between the presence of a *KRAS* mutation and patient survival were examined using Cox regression. For all three survival outcomes, the non-adjusted (crude) effect of the presence of *KRAS* (in tissue as well as cell-free DNA) was examined for association. For OS and RFS, the effect of the *KRAS* mutations after adjustment for potential confounders was examined for association. The CCI had the most impact on results during multivariate analysis; hence, the adjusted variables presented utilise this confounder. Cox regression model estimates are presented as estimate hazard ratios (HRs) with 95% confidence intervals (CIs).

The proportional hazard assumptions were assessed by visual inspection of Kaplan–Meier curves assessing time-dependent covariates and plotting simulated cumulative Martingale residuals of each covariate. The assumption of proportional hazards was deemed appropriate. Statistical analyses were programmed using SPSS v28. A priori, $p < 0.05$ (two-tailed) was used to indicate statistical significance.

3. Results

3.1. KRAS Mutation in ctDNA and Prognosis

Among the 121 patients in this study, there was insufficient plasma for analysis of 41 patients. Insufficient plasma occurred due to the use of plasma samples in another prior study. One further patient had sufficient ctDNA but had incomplete follow-up data so was also excluded. Among the remaining 80 patients, 23 were positive for *KRAS* mutation in ctDNA (28.8%). Patient demographic data and clinical characteristics by ctDNA *KRAS* status are shown in Table 1. The frequency of *KRAS* mutation was found to be significantly higher in non-smokers and those who received adjuvant chemotherapy. All other characteristics were not significantly associated with the *KRAS* mutation status. Interestingly, well and moderately differentiated tumours were the most predominant tumour grade in both *KRAS* negative and *KRAS* positive cases; however, the frequency of poorly differentiated tumours was three times higher in *KRAS* positive cases. Despite these differences, our analysis did not reveal a significant association between *KRAS* status and tumour grade. There was no significant association found between OS, CSS, or RFS in patients who tested positive for ctDNA *KRAS*mut compared to those who were *KRAS*wt (Tables 2 and 3, Figures 1–3). Adjustment for potential confounders produced no statistically significant changes.

Table 1. Demographic and clinical characteristics of patients by ctDNA *KRAS* status.

Characteristic	Response/Statistic	Negative (*n* = 57)	Positive (*n* = 23)	Total (*N* = 80)	*p*-Value Exact
Sex	Male	33 (58%)	9 (39%)	42 (53%)	0.146
	Female	24 (42%)	14 (61%)	38 (48%)	
Smoking status	Non-smoker	30 (53%)	18 (78%)	48 (60%)	0.038
	Ex-smoker	20 (35%)	2 (9%)	22 (28%)	
	Smoker	7 (12%)	3 (13%)	10 (13%)	
Age at operation	mean (SD)	68.91 (13.52)	72.87 (9.28)	70.04 (12.52)	0.374
	median	71.60	73.60	72.23	
BMI	mean (SD)	28.99 (5.81)	28.56 (4.80)	28.87 (5.52)	0.642
	median	27.96	28.10	28.10	
CCI score	mean (SD)	4.91 (1.56)	4.84 (2.31)	4.86 (2.11)	0.429
	median	5	5	5	
Recurrence	No	42 (74%)	15 (65%)	57 (71%)	0.586
	Yes	15 (26%)	8 (35%)	23 (29%)	
Site of cancer	Right	20 (35%)	9 (39%)	29 (36%)	1.0
	Left	34 (60%)	14 (61%)	48 (60%)	
	Synchronous	2 (3%	0	2 (3%)	
	Missing	1 (2%)	0	1 (1%)	
Tumour grade	Well or mod	45 (80%)	15 (65%)	60 (76%)	0.249
	Poorly	4 (7.1%)	5 (22%)	9 (11%)	
	Mucinous or medullary	7 (13%)	3 (13%)	10 (13%)	
	Missing	1	0	1	
Pathological stage	In situ	3 (5.3%)	0	3 (3.8%)	0.853
	Stage 1	13 (23%)	5 (22%)	18 (23%)	
	Stage 2	20 (35%)	7 (30%)	27 (34%)	
	Stage 3	17 (30%)	9 (39%)	26 (33%)	
	Stage 4	4 (7.0%)	2 (8.7%)	6 (7.5%)	
Resection margin	R0	52 (91%)	20 (87%)	72 (90%)	0.830
	R1	2 (4%)	1 (4%)	3 (4%)	
	R2	3 (5%)	2 (9%)	5 (6%)	
Adjuvant chemotherapy	Received	18 (32%)	13 (57%)	31 (39%)	0.046
	Not received	39 (68%)	10 (43%)	49 (61%)	

Table 2. OS, CSS, and RFS by ctDNA *KRAS* status.

		Negative	Positive	Total	Log-Rank
Characteristic	**Response/Statistic**	**(*n* = 57)**	**(*n* = 23)**	**(*N* = 80)**	***p*-Value**
Overall survival *	mean (SD)	6.42 (0.357)	6.30 (0.375)	6.44 (0.295)	0.891
Overall survival **	mean (SD)	4.34 (0.206)	4.67 (0.175)	4.43 (0.155)	0.832
Survival outcome	Censored Death Missing data/Excluded	37 (65%) 16 (28%) 4 (7%)	15 (65%) 6 (26%) 2 (9%)	52 (65%) 22 (27%) 6 (8%)	
Cancer-specific survival *	mean (SD)	6.87 (0.332)	6.44 (0.365)	6.81 (0.282)	0.690
Cancer-specific survival **	mean (SD)	4.49 (0.197)	4.72 (0.175)	4.55 (0.146)	0.747
Survival status	Censored Cancer-specific death Missing data	43 (75%) 10 (18%) 4 (7%)	16 (70%) 5 (22%) 2 (9%)	53 (66%) 21 (26%) 6 (8%)	
Recurrence-free survival *	mean (SD)	6.37 (0.376)	5.12 (0.548)	6.22 (0.331)	0.590
Recurrence-free survival **	mean (SD)	4.26 (0.205)	3.89 (0.367)	4.15 (0.182)	0.616
Survival status	Censored Recurrence Missing data	37 (65%) 14 (25%) 6 (11%)	14 (61%) 7 (30%) 2 (9%)	51 (64%) 21 (26%) 8 (10%)	

* unadjusted follow-up survival data, ** survival adjusted to 5-year endpoints

Table 3. OS, CSS, and RFS hazard ratio by ctDNA *KRAS* status.

	Crude			**Adjusted** [+]		
Model	**HR (95% CI)**	***p*-Value**	***N***	**HR (95% CI)**	***p*-Value**	***N***
Overall survival *	0.94 (0.37, 2.40)	0.891	74	0.97 (0.38, 2.50)	0.952	74
Cancer survival *	1.24 (0.42, 3.65)	0.691	74	1.26 (0.43, 3.71)	0.673	74
Recurrence *	1.28 (0.52, 3.18)	0.591	72	1.28 (0.52, 3.17)	0.598	72
Overall survival **	0.90 (0.35, 2.31)	0.833	74	0.94 (0.37, 2.40)	0.888	74
Cancer survival **	1.19 (0.41, 3.49)	0.748	74	1.21 (0.41, 3.54)	0.731	74
Recurrence **	1.26 (0.51, 3.12)	0.617	72	1.26 (0.51, 3.11)	0.623	72

[+] Adjustment for CCI only displayed in this example, * unadjusted follow-up survival data, ** survival adjusted to 5-year endpoints.

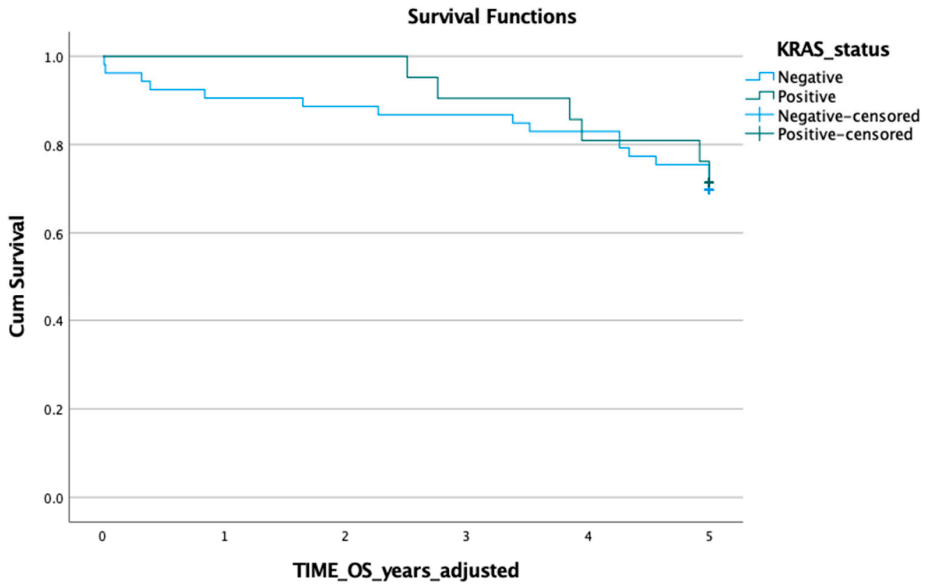

Figure 1. Kaplan–Meier curve of OS vs. ctDNA *KRAS* status.

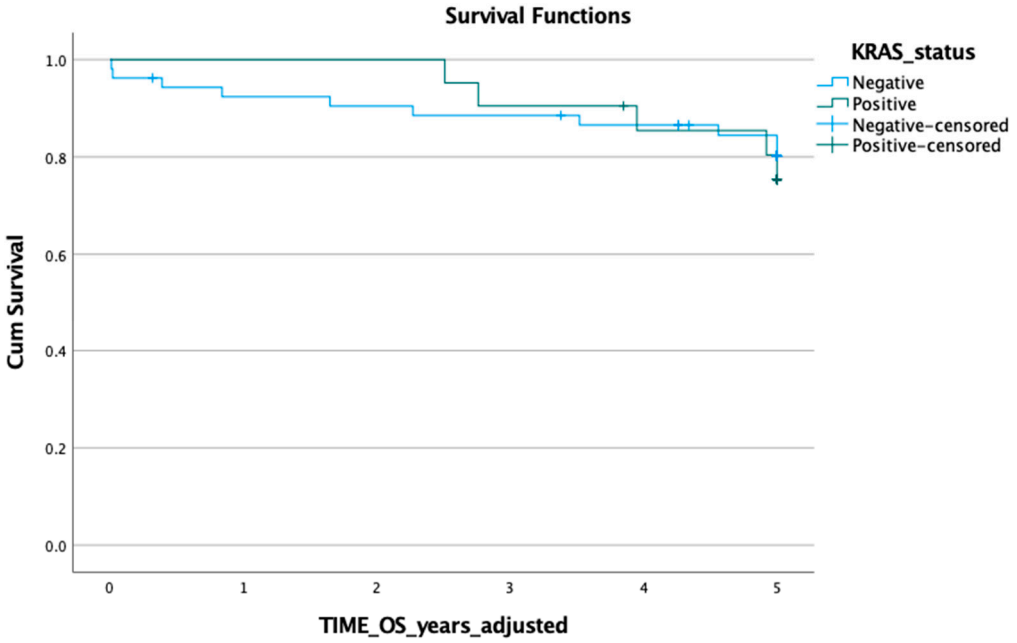

Figure 2. Kaplan–Meier curve of CSS vs. ctDNA *KRAS* status.

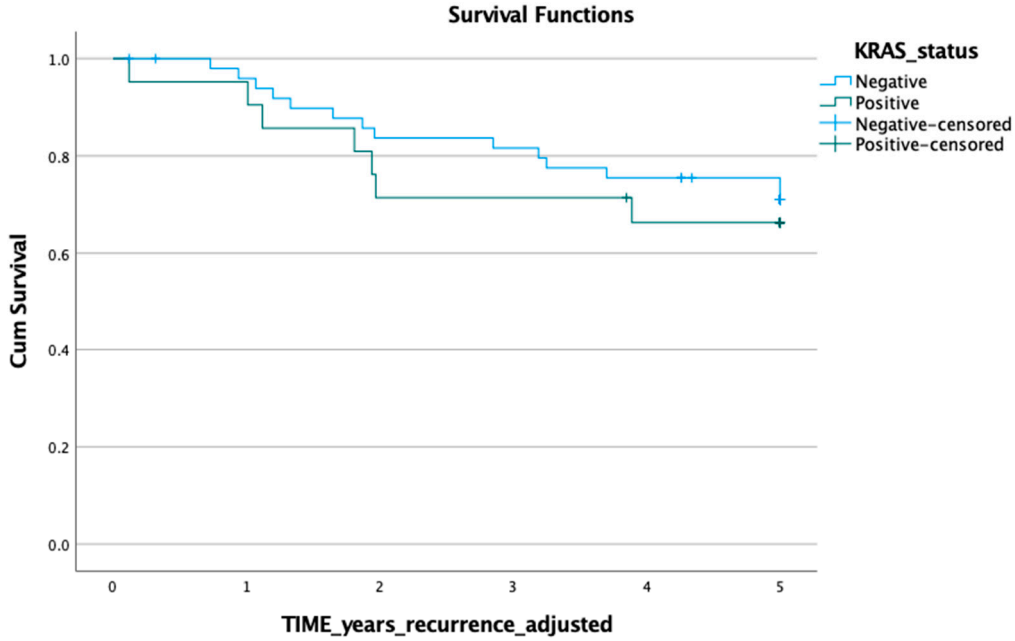

Figure 3. Kaplan–Meier curve of RFS vs. ctDNA *KRAS* status.

3.2. KRAS Mutation in Tumour Tissue and Prognosis

Tumour tissue was available for analysis from 107 patients. Amongst these patients, 78 were positive for *KRAS* mutation in tumour tissue DNA when using the *LoD* cut-off (72.9%). This rate decreased sequentially with cut-off values of 1%, 5%, or 10% MAF, which resulted in frequencies of 24.3%, 22.4%, and 21.5%, respectively. Patient demographic data and clinical characteristics by tissue *KRAS* status are shown in Table 4. The frequency of *KRAS* mutation was not significantly different in any of these factors. There was no significant association found between OS, CSS, or RFS in patients who tested positive for tumour tissue DNA *KRAS*mut compared to those who were *KRAS*wt (Table 5, Figures 4–6). However, there was a trend towards decreased overall, cancer-specific, and recurrence-free survival for tumour tissue *KRAS*mut positive cases. The same non-statistically significant

trend was seen when analysis was performed using a >10 MAF cut-off point (Table 6). Similarly, although it is not consistent across every model, there was a trend towards an increased HR for all these outcomes (Table 7). Adjustment for confounders produced no statistically significant changes.

Table 4. Demographic and clinical characteristics of patients by tissue DNA *KRAS* status.

		Negative	Positive	Total	*p*-Value Exact
Characteristic	**Response/Statistic**	**(*n* = 29)**	**(*n* = 78)**	**(*N* = 107)**	
Sex	Male	15 (52%)	45 (58%)	60 (56%)	0.663
	Female	14 (48%)	33 (42%)	47 (44%)	
Smoking status	Non-smoker	19 (66%)	48 (62%)	67 (63%)	0.286
	Ex-smoker	5 (17%)	23 (29%)	28 (26%)	
	Smoker	5 (17%)	7 (9%)	12 (11%)	
Age at operation	mean (SD)	65.66 (14.18)	71.32 (11.38)	69.78 (12.39)	0.623
	median	65.60	73.55	71.60	
BMI	mean (SD)	27.96 (6.22)	28.49 (4.91)	28.34 (5.28)	0.346
	median	28.88	27.30	27.75	
CCI score	mean (SD)	4.48 (1.7)	5.42 (1.96)	5.17 (1.93)	0.531
	median	4	5	5	
Recurrence	No	24 (83%)	59 (76%)	83 (78%)	0.603
	Yes	5 (17%)	19 (24%)	24 (22%)	
Site of cancer	Right	8 (28%)	32 (41%)	40 (37%)	0.444
	Left	21 (72%)	43 (55%)	64 60%)	
	Synchronous	0	2 (3%)	2 (2%)	
	Missing	0	1 (1%)	1 (1%)	
Tumour grade	Well or mod	23 (79%)	59 (77%)	82 (77%)	1.0
	Poorly	2 (6.9%)	7 (9.1%)	9 (8.5%)	
	Mucinous or medullary	4 (14%)	11 (14%)	15 (14%)	
	Missing	0	1	1	
Pathological stage	In situ	3 (10%)	2 (2.6%)	5 (4.7%)	0.413
	Stage 1	6 (21%)	19 (24%)	25 (23%)	
	Stage 2	7 (24%)	26 (33%)	33 (31%)	
	Stage 3	12 (41%)	26 (33%)	38 (36%)	
	Stage 4	1 (3.4%)	5 (6.4%)	6 (5.6%)	
Resection margin	R0	28 (97%)	73 (94%	101 (94%)	0.829
	R1	0	2 (3%)	2 (2%)	
	R2	1 (3%)	3(4%)	4 (4%)	
Adjuvant chemotherapy	Received	14 (48%)	27 (35%)	41 (38%)	0.263
	Not received	15 (52%)	51 (65%)	66 (62%)	

Table 5. OS, CSS, and RFS by tissue DNA *KRAS* status.

		Negative	Positive	Total	Log-Rank
Characteristic	**Response/Statistic**	**(*n* = 29)**	**(*n* = 78)**	**(*N* = 107)**	***p*-Value**
Overall survival time *	mean (SD)	6.45 (0.352)	6.22 (0.321)	6.42 (0.264)	0.251
Overall survival time **	mean (SD)	4.61 (0.218)	4.23 (0.179)	4.34 (0.142)	0.193
Survival outcome	Censored Death	22 (76%) 6 (21%)	48 (62%) 26 (33%)	70 (65%) 32 (30%)	
Cancer-specific survival time *	mean (SD)	6.71 (0.319)	6.73 (0.303)	6.87 (0.248)	0.411
Cancer-specific survival time **	mean (SD)	4.71 (0.215)	4.41 (0.168)	4.50 (0.132)	0.312
Survival status	Censored Cancer-specific death Missing data/excluded	24 (83%) 4 (14%) 1 (3%)	57 (73%) 17 (22%) 4 (5%)	81 (76%) 21 (20%) 5 (5%)	
Recurrence-free survival time *	mean (SD)	6.27 (0.418)	6.46 (0.345)	6.59 (0.284)	0.439
Recurrence-free survival time **	mean (SD)	4.41 (0.251)	4.16 (0.196)	4.23 (0.158)	0.443
Survival status	Censored Recurrence Missing data/excluded	22 (76%) 5 (17%) 2 (7%)	54 (69%) 18 (23%) 6 (8%)	76 (71%) 23 (22%) 8 (7%)	

* Unadjusted follow-up survival data, ** survival adjusted to 5-year endpoints.

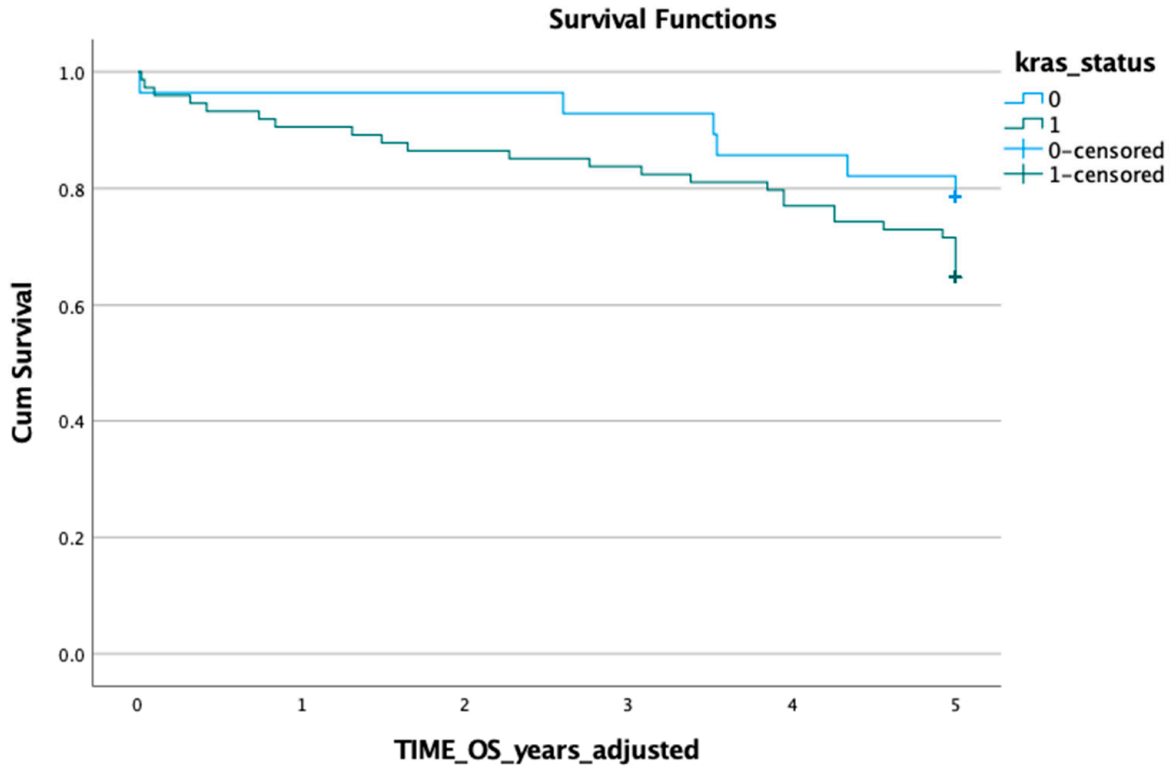

Figure 4. Kaplan–Meier curve of OS vs. tissue DNA *KRAS* status.

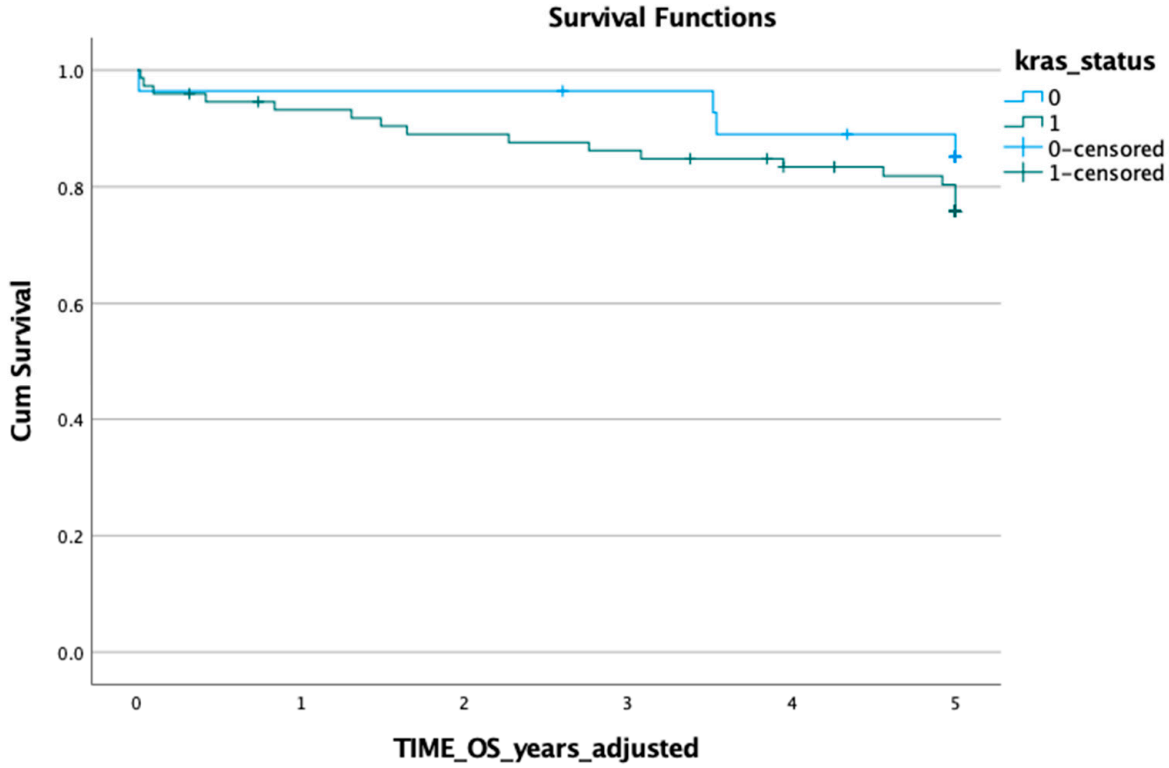

Figure 5. Kaplan–Meier curve of CSS vs. tissue DNA *KRAS* status.

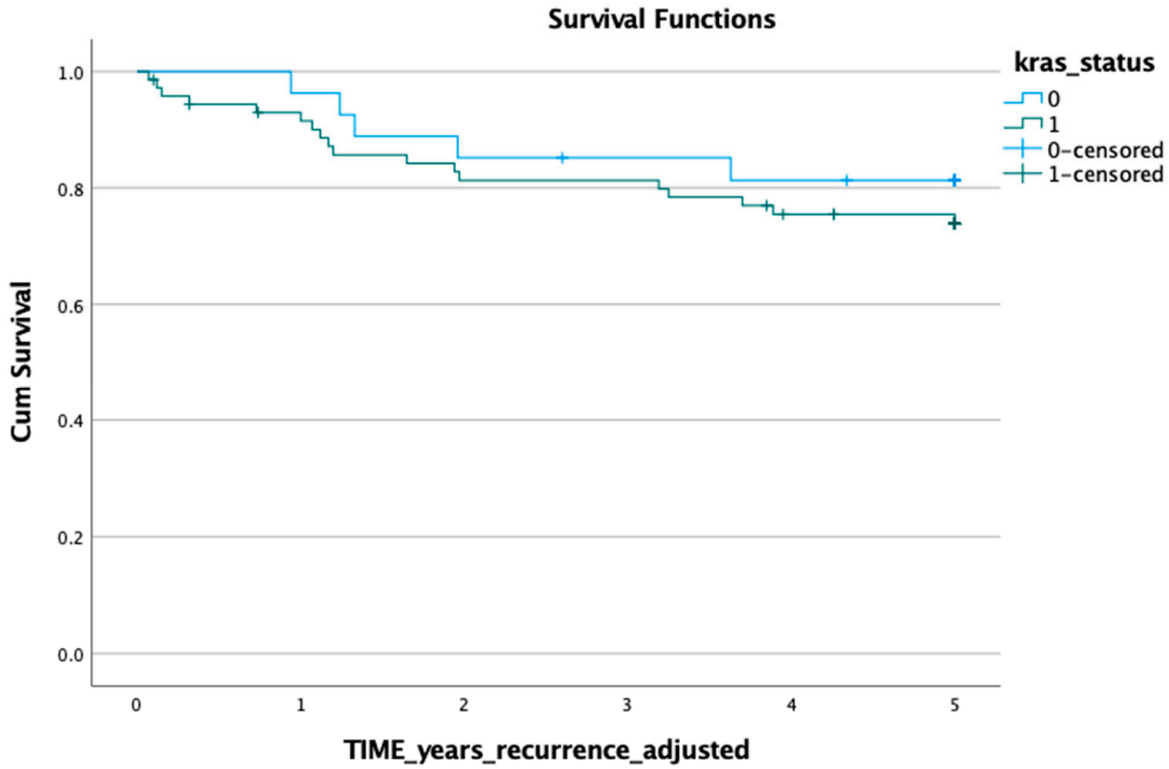

Figure 6. Kaplan–Meier curve of RFS vs. tissue DNA *KRAS* status.

Table 6. OS, CSS, and RFS by tissue DNA *KRAS* > 10MAF status.

		Negative	**Positive**	**Total**	**Log-Rank**
Characteristic	**Response/Statistic**	**(*n* = *84*)**	**(*n* = *23*)**	**(*N* = *107*)**	***p*-Value**
Overall survival time *	mean (SD)	6.46 (0.280)	6.12 (0.607)	6.12 (0.264)	0.628
Overall survival time **	mean (SD)	4.39 (0.154)	4.14 (0.365)	4.34 (0.142)	0.544
Survival outcome	Censored	56 (67%)	14 (61%)	70 (65%)	
	Death	24 (29%)	8 (35%)	32 (30%)	
	Missing data/excluded	4 (5%)	1 (4%)	5 (5%)	
Cancer-specific survival time *	mean (SD)	6.95 (0.251)	6.43 (0.606)	6.87 (0.248)	0.476
Cancer-specific survival time **	mean (SD)	4.58 (0.138)	4.19 (0.376)	4.50 (0.132)	0.382
Survival status	Censored	65 (77%)	16 (70%)	81 (76%)	
	Cancer-specific death	15 (18%)	6 (26%)	21 (20%)	
	Missing data/excluded	4 (5%)	1 (4%)	5 (5%)	
Recurrence-free survival time *	mean (SD)	6.54 (0.306)	6.43 (0.660)	6.59 (0.284)	0.914
Recurrence-free survival time **	mean (SD)	4.28 (0.173)	4.06 (0.373)	4.23 (0.158)	0.924
Survival status	Censored	60 (71%)	16 (70%)	76 (71%)	
	Recurrence	18 (21%)	5 (22%)	23 (22%)	
	Missing data/excluded	6 (7%)	2 (9%)	8 (7%)	

* Unadjusted follow-up survival data, ** survival adjusted to 5-year endpoints.

Table 7. OS, CSS, and RFS hazard ratio by tissue DNA *KRAS* status.

		Crude			**Adjusted** [+]		
Model	**Cut-Off Method**	**HR (95% CI)**	***p*-Value**	***N***	**HR (95% CI)**	***p*-Value**	***N***
OS *	*LoD*	1.68 (0.69, 4.09)	0.257	102	1.48 (0.60, 3.67)	0.395	102
CSS *		1.58 (0.53, 4.73)	0.415	102	1.49 (0.49, 4.53)	0.485	102
RFS *		1.48 (0.55, 3.98)	0.441	99	1.43 (0.52, 3.91)	0.488	99
OS *	>10% MAF	1.22 (0.55, 2.73)	0.628	102	1.04 (0.45, 2.36)	0.934	102
CSS *		1.42 (0.54, 3.69)	0.475	102	1.32 (0.49, 3.51)	0.581	102
RFS *		1.06 (0.39, 2.84)	0.914	99	0.99 (0.36, 2.75)	0.990	99
OS *	>5% MAF	1.41 (0.65, 3.06)	0.387	102	1.21 (0.55, 2.67)	0.643	102
CSS *		1.74 (0.70, 4.35)	0.237	102	1.64 (0.64, 4.19)	0.305	102
RFS *		1.34 (0.53, 3.39)	0.540	99	1.28 (0.49, 3.34)	0.617	99
OS *	>1% MAF	1.25 (0.57, 2.71)	0.576	102	1.10 (0.50, 2.41)	0.820	102
CSS *		1.55 (0.62, 3.87)	0.349	102	1.46 (0.58, 3.71)	0.422	102
RFS *		1.17 (0.46, 2.97)	0.743	99	1.12 (0.43, 2.89)	0.817	99
OS **	*LoD*	1.78 (0.73, 4.33)	0.202	102	1.56 (0.63, 3.84)	0.336	102
CSS **		1.74 (0.58, 5.16)	0.320	102	1.61 (0.53, 4.86)	0.399	102
RFS **		1.47 (0.55, 3.96)	0.446	99	1.42 (0.52, 3.90)	0.491	99
OS **	>10% MAF	1.28 (0.57, 2.85)	0.548	102	1.05 (0.46, 2.40)	0.900	102
CSS **		1.52 (0.59, 3.92)	0.387	102	1.37 (0.52, 3.64)	0.526	102
RFS **		1.05 (0.39, 2.83)	0.924	99	0.99 (0.36, 2.74)	0.983	99
OS **	>5% MAF	1.47 (0.68, 3.18)	0.326	102	1.23 (0.56, 2.72)	0.612	102
CSS **		1.86 (0.75, 4.60)	0.182	102	1.70 (0.67, 4.34)	0.268	102
RFS **		1.33 (0.52, 3.73)	0.548	99	1.27 (0.49, 3.32)	0.624	99
OS **	>1% MAF	1.29 (0.60, 2.78)	0.522	102	1.11 (0.50, 2.43)	0.802	102
CSS **		1.63 (0.66, 4.03)	0.294	102	1.50 (0.59, 3.79)	0.390	102
RFS **		1.16 (0.46, 2.94)	0.757	99	1.11 (0.43, 2.87)	0.829	99

[+] Adjustment for CCI only displayed in this example, * unadjusted follow-up survival data, ** survival adjusted to 5-year endpoints, MAF = mutation allele frequency, *LOD* = limit of detection.

4. Discussion

The results show a trend towards increased risk of disease recurrence and decreased OS and CSS for patients with *KRAS* mutation found in their tumour tissue. However, these trends were not statistically significant in univariate or multivariate analyses. It is possible that this study was underpowered; however, as previously demonstrated, the number of participants with tissue samples in this study (n = 107) was above the median number of participants (n = 97) found after a review of the literature when searching for articles analysing the prognostic utility of *KRAS* mutations in CRC tumour tissue or plasma (Chapter 1: Background—Table 1). Regarding features such as the TNM stages included the following: receipt of adjuvant chemotherapy, type of chemotherapy received, type of PCR method used, and methods of survival analysis; the heterogeneity of the research found illustrates the difficulty in comparing the current evidence in this field.

Our results show a non-significant trend towards an increased risk of disease recurrence, a decreased overall survival, and a decreased cancer-specific survival for patients with *KRAS* mutation-positive tissue at the time of diagnosis and initial treatment. These results were not reiterated in ctDNA, which failed to show any trend or significant association between *KRAS* mutation status and overall survival, cancer-specific survival, or recurrence-free survival.

The number of patients found to have *KRAS*mut-positive tissue samples was well above the expected range of 20% to 40% [33]. However, if the MAF cut-offs of 1%, 5%, and 10% are used, then the frequency falls within the expected range of 21–25%. Despite the higher *KRAS*mut clonal population of cells present in the tumours with these cut-offs, this did not translate into any significant changes in survival or recurrence (Tables 6 and 7).

The limitations of this study are its relatively small sample size, retrospective nature, and lack of complete standardization of patient care and plasma collection methods. The patients were managed clinically at the discretion of the treating surgeon and oncologist, and CEA was not routinely performed for all patients, hence there were insufficient numbers to include this marker in the analysis. The slight differences in the collection of the plasma samples potentially could have affected the overall frequency of ctDNA *KRAS* mutations. However, since the classification of *KRAS* mutation status was based on the *LoD*, this should be unaffected by potential variances in cell-free DNA release at the time of collection due to different sample types. Furthermore, ddPCR has been found to have a high resistance to the effect of potential PCR inhibitors, and Sefrioui et al. found that heparinase treatment of samples did not quantitatively or qualitatively alter the ctDNA detection [34–36]. Finally, although the sample size is small, it is comparable to other studies in this area and there is a significantly longer follow-up time for this cohort. The sample size was insufficient to perform subgroup analyses for the *KRAS* status stratified by either adjuvant therapy, site of cancer, or resection margins, which are all known to have effects on oncological outcomes.

Similarly, the sample size was insufficient to perform subgroup analyses based on the specific *KRAS* mutation type. This is in addition to the fact that the specific assay kit used was not able to sufficiently differentiate the mutation types to allow for certainty. There is mixed evidence regarding the effect that specific codon mutations have on prognoses. Jones et al. and Imamura et al. found that the G12C and G12V mutations conferred a worse prognosis compared to other mutations in codons 12, 13, or 61 [37,38]. However, a pooled analysis of patients from five trials found conflicting results that suggested there was a similarly poorer prognosis with G12C mutations but that G12D and G12V mutations had no obvious impacts on OS in univariate and multivariate analyses [39]. These studies were all performed on tissue samples, which highlights that there is still ongoing debate about the prognostic utility of *KRAS* mutations for CRC even when the mutational analysis is performed on the primary cancer itself. Studies that have found an association between *KRAS* mutations and OS are more likely to have recruited patients with metastatic colorectal cancer rather than early-stage cancer. For instance, Mendoza-Moreno et al. found that at 36 months more patients with peritoneal metastases and *KRAS*wt tumours were alive

compared to *KRAS*mut (31% vs. 15%; $p < 0.001$) [40]. Alkader et al. found a similar association of shorter overall survival in *KRAS*mut when compared to *KRAS*wt patients (21 months vs. 17 months) [41].

The poor concordance (44%) between ctDNA and tumour tissue *KRAS* mutation status in matched patient samples suggests the limited feasibility of ctDNA as a useful biomarker (Table 8). However, the situation is complex and the reason for this discordance is potentially multifactorial. Firstly, the accuracy and sensitivity of ctDNA seem to increase with the overall burden of disease. Several small and large studies have found that the diagnostic accuracy of ctDNA is related to the stage of disease, and, therefore, whilst early-stage tumours could harbor *KRAS* mutations, these are less likely to be simultaneously found in ctDNA when compared to stage III or IV cancers [20,42–44]. This pattern explains why many of the studies utilising ctDNA focused only on metastatic CRC [22,25–29]. Furthermore, the heterogeneity of genetic mutations within CRC means that in many of the tissue DNA samples, there is likely to be a small sub-clonal population of cells with *KRAS* mutations. This fractional volume of *KRAS* mutated cancer cells may not shed enough DNA into the circulation to produce a positive result in the plasma whilst still being sufficient to be detected at low volumes in the tumour tissue due to the high sensitivity of ddPCR. This is supported by the fact that the frequency of *KRAS* mutations drops from 72.9% to 21.5% if a cut-off of an MAF > 10% is used rather than the *LoD* methodology. Similarly, the concordance between ctDNA and tumour tissue also increases from 44% to 73% with this change in cut-off value.

Table 8. Concordance between ctDNA and tissue *KRAS* status.

		ctDNA		
		Positive	**Negative**	**Total**
Tumour tissue (*LoD* Cut-off)	Positive	15	34	49
	Negative	3	14	17
	Total	18	48	66
Tumour tissue (10% MAF Cut-off)	Positive	6	6	12
	Negative	12	42	54
	Total	18	48	66

Although this study is not designed to answer any questions regarding the clinical significance of the detection of low-volume *KRAS* mutations, it is likely that this is related to the emergence of acquired resistance to targeted epidermal growth factor receptor (EGFR) blockade therapy [45–48]. The use of anti-EGFR therapy in CRC is limited to patients with a *KRAS* mutation-negative status (generally a MAF < 5% on tumour tissue) since it has been shown that this treatment has a reduced effect on OS, CSS, or RFS in patients harbouring a *KRAS* mutation [49]. However, in this cohort, around 50% of the cases were found to harbour low-frequency *KRAS* mutations that would lead to the development of treatment resistance. There is evidence that the emergence of resistance to anti-EGFR treatment can be overcome if combined with other therapies simultaneously, such as MEK pathway inhibition [50,51]. Therefore, the identification of low-frequency *KRAS* mutations in patients who qualify for anti-EGFR therapy could be an indication for dual first-line therapy with treatment such as MEK inhibitors and is an area worth investigating but is beyond the scope of this study.

5. Conclusions

Our results show a non-significant trend towards an increased risk of disease recurrence, decreased overall survival, and decreased cancer-specific survival for patients with *KRAS* mutation-positive tissue at the time of diagnosis and initial treatment. These results were not reiterated in ctDNA, which failed to show any significant association between *KRAS* mutation status and overall survival, cancer-specific survival, or recurrence-free survival.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/gastroent15010008/s1, Supplementary Material S1—Methods of plasma separation and genomic DNA extraction.

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Data Availability Statement: Part of the data presented in this study are available within the article and Supplementary Material S1 file. The rest of the data presented in this study are available on request to the corresponding author. The data are not publicly available due to ethics approval restrictions and privacy reasons for the participants in this study.

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