Ki-67 index and response to chemotherapy in patients with neuroendocrine tumours

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Abstract

Chemotherapy (CT) is widely used for neuroendocrine tumours (NETs), but there are no validated biomarkers to predict response. The Ki-67 proliferation index has been proposed as a means of selecting patients for CT, but robust data are lacking. The aim of this study was to investigate the relationship between response to chemotherapy and Ki-67 in NET. We reviewed data from 222 NET patients treated with CT. Tumours were graded according to Ki-67 index: G1 ≤2%, G2 3–20% and G3 >20%. Response was assessed according to RECIST and survival calculated from start of chemotherapy to death. To explore Ki-67 as a marker of response, we calculated the likelihood ratio and performed receiver operating characteristic analysis. Overall, 193 patients had a documented Ki-67 index, of which 173 were also evaluable for radiological response: 10% were G1, 46% G2 and 43% G3; 46% were pancreatic NET (PNET). Median overall survival was 22.1 months. Overall response rate was 30% (39% in PNET vs 22% in non-PNET) and 43% of patients had stable disease. Response rate increased with grade: 6% in G1 tumours, 24% in G2 and 43% in G3. However, maximum likelihood ratio was 2.3 at Ki-67 = 35%, and the area under the ROC curve was 0.60. As reported previously, a high Ki-67 was an adverse prognostic factor for overall survival. In conclusion, response to CT increases with Ki-67 index, but Ki-67 alone is an unreliable means to select patients for CT. Improved methods to stratify patients for systemic therapy are required.

Key Words
- neuroendocrine tumour
- chemotherapy
- Ki-67
- response

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Introduction

Neuroendocrine tumours (NETs) are exceptional in terms of their heterogeneity with respect to clinical behaviour and prognosis. Patients may live with low-grade, indolent tumours for 20 years while the outlook for high-grade tumours can be as little as 6 months (Yao *et al.* 2008). Although still considered a rare disease entity, the incidence of NET is increasing and the prevalence is now greater than that of many other tumours of the

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gastrointestinal tract (Yao *et al.* 2008). They therefore represent a significant public health burden.

The majority of NET patients present with metastatic disease and are not suitable for surgical resection with curative intent. In these cases, palliative treatments are required with the aim of controlling symptoms, delaying disease progression and improving survival, and a range of therapeutic interventions are now available. Somatostatin analogues (SSAs) have been used for many years to control symptoms associated with well-differentiated functional tumours, and recent studies have also demonstrated an anti-proliferative effect resulting in delayed progression rather than tumour response (Rinke *et al.* 2009, Caplin *et al.* 2014). Radiolabelled SSAs using lutetium (Kwekkeboom *et al.* 2008) and yttrium (Imhof *et al.* 2011, Villard *et al.* 2012) have also been widely used and recently shown to have a significantly improved progression-free survival (PFS) in patients with progressive disease on a fixed dose of SSA at baseline compared with high dose SSA alone (Strosberg 2015). For pancreatic NET (PNET), sunitinib and everolimus have been shown to delay progression and are associated with response rates of 9 and 5%, respectively (Raymond *et al.* 2011, Yao *et al.* 2011). Cytotoxic chemotherapy is the standard of care for poorly differentiated, high-grade tumours, but the overall prognosis is poor despite its use. Additionally, streptozocin-based chemotherapy has an established role in the management of PNET based on the seminal studies of Moertel and coworkers, which demonstrated an improvement in survival for multi-agent therapy along with radiological, clinical and biochemical responses (Moertel *et al.* 1980, 1992). Recent studies applying RECIST or WHO criteria alone report response rates for PNET varying between 17 and 38% (Delaunoit *et al.* 2004, Kouvaraki *et al.* 2004, Turner *et al.* 2010,

Table 1 Patient characteristics.

Characteristics	All patients *n* (%) *n* = 173	Pancreatic *n* (%) *n* = 79	Non-pancreatic *n* (%) *n* = 94
Age (median (range))	58 (22–84)	55 (23–83)	60 (22–84)
Gender			
Male	97 (56.1)	43 (54.4)	54 (57.4)
Female	76 (43.9)	36 (45.6)	40 (42.6)
Treatment			
Platinum-etoposide	27 (15.6)	8 (10.1)	19 (20.2)
Streptozocin-Fluoropyrimidine	21 (12.1)	9 (11.4)	12 (12.8)
Streptozocin-Fluoropyrimidine-platinum	125 (72.3)	62 (78.5)	63 (67.0)
Differentiation			
Well	89 (51.4)	44 (55.7)	45 (47.0)
Poor	64 (37.0)	23 (29.1)	41 (43.6)
Unknown	20 (11.6)	12 (15.2)	8 (8.5)
Grade			
(ENETS)			
G1 (Ki-67 ≤2%)	18 (10.4)	9 (11.4)	9 (9.6)
G2 (Ki-673-≤20%)	80 (46.2)	40 (50.6)	40 (42.6)
G3 (Ki-67 >20%)	75 (43.4)	3 (38.0)	45 (47.9)
Primary site			
Midgut	23 (13.3)	–	23 (24.5)
Unknown	33 (19.1)	–	33 (35.1)
Hindgut	19 (11.0)	–	19 (20.2)
Lung	16 (9.2)	–	16 (17.0)
Pancreas	79 (45.7)	79 (100)	–
Other	3 (1.7)	–	3 (3.2)
Octreotide scan			
Positive	94 (54.3)	51 (64.6)	43 (45.7)
Negative	33 (19.1)	9 (11.4)	24 (25.5)
Unknown	46 (26.6)	19 (24.1)	27 (28.7)
Performance status			
0	45 (26.0)	22 (27.8)	23 (24.5)
1	102 (59.0)	45 (57.0)	57 (60.6)
2	17 (9.8)	7 (8.9)	10 (10.6)
3	6 (3.5)	2 (2.5)	4 (4.3)
4	1 (0.6)	1 (1.3)	0
Unknown	2 (1.2)	2 (2.5)	0

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Weatherstone & Meyer 2012, Meyer *et al.* 2014, Clewemar Antonodimitrakis *et al.* 2015, Dilz *et al.* 2015, Krug *et al.* 2015), while non-PNET appears to have a lower response rate of 16% (Engstrom *et al.* 1984, Sun *et al.* 2005).

A key challenge for clinicians is to define the optimal therapeutic algorithm for such diverse tumours with a range of therapeutic options. While SSA therapy is usually reserved for somatostatin receptor-positive tumours, we have no reliable predicative markers to select patients for sunitinib, everolimus or chemotherapy. The proliferation marker, Ki-67, has been suggested as a means to select patients for chemotherapy and a threshold for Ki-67 of 10% has been proposed but no data provided to support this (Vilar *et al.* 2007). A recent study in high-grade tumours defined a threshold Ki-67 index of 55%, above which the response rate was 42% compared with 14% for those below 55% (Sorbye *et al.* 2013). However, chemotherapy clearly has a role in lower grade tumours, and we have previously demonstrated a correlation between grade and response in these categories (Turner *et al.* 2010). We have therefore analysed a large multicentre cohort of NET patients treated with chemotherapy to explore the validity of Ki-67 as a robust method of stratifying patients for chemotherapy.

Materials and methods

We identified 222 NET patients treated with chemotherapy between May 1999 and June 2015 from the following three ENETS Centres of Excellence in the UK: The Royal Free London, NHS Foundation Trust, London, UK; The Christie NHS Foundation Trust, Manchester; and King's College Hospital, London, UK. Patients were required to have a histological diagnosis of NET or NEC and be receiving first-line chemotherapy with palliative intent. No specific chemotherapy regimens were excluded, as first-line palliative regimens differed slightly across the three clinical sites. Data on the regimen received were collected for all patients and reported in Table 1.

Two independent radiologists assessed response according to RECIST 1.0. CT scans were performed at baseline (within 6 weeks of commencing chemotherapy) and after three cycles of treatment. In the absence of disease progression or unacceptable toxicity, patients went on to complete a total of six cycles with repeat imaging at this time. Thereafter, they were scanned at three monthly intervals until radiological progression of disease was confirmed. Overall (OS) and progression free survival (PFS) were defined as the interval between the start of chemotherapy and death or radiological progression, as assessed by independent review.

Ki-67 immunohistochemical staining was performed with the NovoLink Polymer detection system (Novocastra, Newcastle-upon-Tyne, UK) (Khan *et al.* 2013) and patients were categorised according to the European Neuroendocrine Tumour Society (ENETS) grading system (Rindi *et al.* 2006, 2007).

Statistics

The statistical analysis was performed using SPSS version 22.0. Kaplan–Meier survival analysis was used to calculate median duration of response, time to progression and OS. Fisher's exact test and χ^2 test for trend were used for predictive markers of response to chemotherapy. Cox regression analyses were used to test the influence of baseline characteristics of grade, ECOG performance status (PS) and primary site on PFS and OS. Hazard ratios and 95% confidence intervals were calculated. Receiver operating characteristic (ROC) curve analysis was used to define the best cut-off value for Ki-67 index with regards to response rate (response = complete response (CR) + partial response (PR), no response = stable disease (SD) + progressive disease (PD)).

Results

Patient characteristics

We reviewed 222 patients in total, of whom 193 had Ki-67 data, and 173 were evaluable for radiological

Table 2 Response and baseline characteristics.

Baseline characteristic	Non-responder	Responder	*P*-value
Primary site			
Non-pancreatic	73 (77.7)	21 (22.3)	0.016
Pancreatic	48 (60.8)	31 (39.2)	
Ki-67			
ENETS			
Ki-67 ≤2%	17 (94.4)	1 (5.6)	0.002
Ki-67 3–≤20%	61 (73.3)	19 (23.8)	
Ki-67 >20%	43 (57.3)	32 (42.7)	
Differentiation			
Well	67 (75.3)	22 (24.7)	0.036
Poor	38 (59.4)	26 (40.6)	
Octreotide scan			
Negative	23 (69.7)	10 (30.3)	0.953
Positive	65 (69.1)	29 (30.9)	
Age			
<58	58 (68.6)	27 (31.4)	0.703
≥58	62 (71.3)	25 (28.7)	
Performance status			
0	30 (66.7)	15 (33.3)	0.712
1	74 (72.5)	28 (27.5)	
2–4	16 (66.7)	8 (33.3)	

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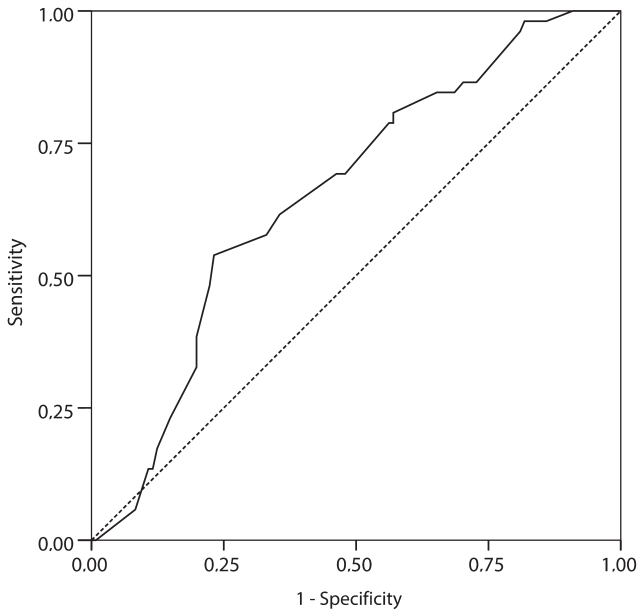

Figure 1
ROC analysis of Ki-67 with regards to response rate.

response and Ki-67 index. The patient characteristics are shown in Table 1. Overall, 43% had G3 grade tumours, but for both pancreatic and non-pancreatic primaries, the majority of patients were classified as G1 or G2 grade according to Ki-67 index. The most common non-PNET sites were the GI tract and lung, but 19% had an unknown primary site. Patients with PNET were more likely to have positive octreotide scans than non-PNET (65% vs 46%, $P=0.008$). First-line chemotherapy was given with platinum/etoposide ($n=27$), streptozocin/fluoropyrimidine ($n=21$) or streptozocin/fluropyrimidine/platinum ($n=125$) and the median number of chemotherapy cycles received was 6 with 58% completing the full course of 6 cycles.

Response

In the 173 patients evaluable, the overall response rate was 30%. Stable disease was the best response in 48%, and 22% had disease progression. Among those with pancreatic primaries, the response rate was 39% (31 out of 79 patients) compared with 22% (21 out of 94 patients) for non-pancreatic tumours ($P=0.02$, Fisher's exact test). Of those patients who had stable disease, 79% (60 out of 83 patients) remained progression free at 6 months. The median duration of response was 7.5 months.

There was evidence that response to chemotherapy was associated with grade defined by Ki-67 using the

Table 3 Response and Ki-67.

Ki-67 (%)	DR	FPR	LR
≥5	96.1	81.0	1.19
≥10	84.6	65.3	1.29
≥15	78.8	56.2	1.40
≥20	69.2	46.3	1.49
≥25	61.5	35.5	1.73
≥30	57.7	33.1	1.74
≥35	53.8	23.1	2.33
≥40	48.1	22.3	2.16
≥45	38.5	19.8	1.94
≥50	32.7	19.8	1.65
≥55	23.1	14.9	1.55
≥60	23.1	14.9	1.55

DR, detection rate; FPR, false-positive rate; LR likelihood ratio.

ENETS criteria cut-off of 20% between G2 and G3, with a response rate of 20% for G1–2 and 43% for G3 ($P=0.002$ Fisher's and $P=0.002$ chi-squared test for trend, respectively; Table 2). In order to explore the ability of Ki-67 to reliably predict response, we performed ROC analysis. The area under the ROC curve (Fig. 1) was 0.66 (95% CI: 0.57–0.74) implying a 66% chance that a randomly chosen responder would have a higher Ki-67 value than a randomly chosen non-responder. As demonstrated in Table 3, there are no cut-off points which combine satisfactory detection and false-positive rates. The maximum likelihood ratio was 2.33 at a cut-off of ≥35%, corresponding to a false-positive rate of 24% (predicting a response in a patient who does not respond) and a modest detection rate of 54% (correctly predicting a response). Response rate was lower in

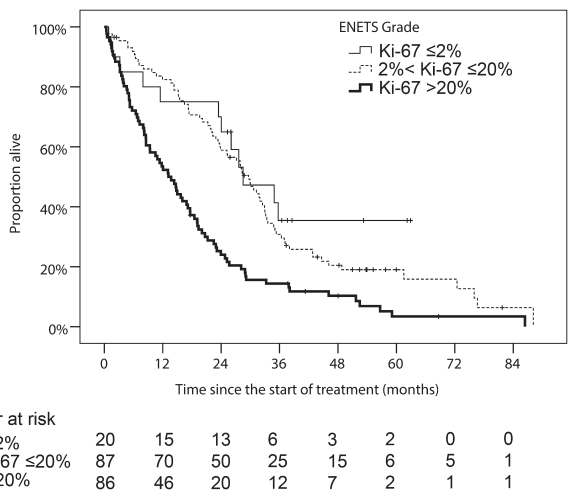

Figure 2
Overall survival stratified by grade as defined by Ki-67 index using ENETs grading.

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Table 4 Overall survival and baseline characteristics.

	Univariate analysis			Multivariable analysis ($n=190$)		
Baseline characteristic	Events/*n*	OS HR (95% CI)	*P*-value	Events/*n*	OS HR (95% CI)	*P*-value
Primary site ($n=193$)						
Non-pancreatic	91/106	1.00	0.006	90/105	1.00	0.052
Pancreatic	74/87	0.65 (0.48, 0.89)		72/85	0.73 (0.53–1.00)	
Ki-67 ($n=193$)						
ENETS grade						
Ki-67 ≤2%	12/20	1.00	<0.001	12/20	1.00	<0.001
Ki-673-≤20%	72/87	1.32 (0.71, 2.44)		69/84	1.23 (0.66–2.30)	
Ki-67 >20%	81/86	2.58 (1.40, 4.76)		81/86	2.61 (1.39–4.90)	
Octreotide scan ($n=135$)						
Negative	37/39	1.00	0.001			
Positive	83/96	0.51 (0.34, 0.75)				
Age ($n=193$)						
<58	82/93	1.00	0.067	80/91	1.00	0.038
≥58	83/100	1.33 (0.98, 1.81)		82/99	1.40 (1.02–1.93)	
Performance status ($n=190$)						
0	42/48	1.00	0.004	42/48	1.00	0.027
1	90/110	1.20 (0.83, 1.73)		90/110	1.20 (0.82–1.74)	
2–4	30/32	2.24 (1.39, 3.61)		30/32	2.01 (1.23–3.31)	
Treatment ($n=193$)						
Doublets	47/59	1.00	0.032	44/56	1.00	0.54
Streptozocin-Fluoropyrimidine-platinum	118/134	0.69 (0.49–0.97)		118/134	0.89 (0.62–1.29)	

Octreotide scan result not included in multivariate analysis due to missing data.

tumours with Ki-67 <35% as compared with those with Ki-67 ≥35% (21% vs 50%; $P<0.001$), and 68% of all tumours had a Ki-67 <35%. Both the ROC curve and likelihood ratio analysis demonstrated that Ki-67 alone is not an accurate predictor of response.

Survival

Patients were followed up for a median of 55 months (range 0.88–88 months). The median OS was 22.1 months (95% CI: 18.7–25.5) (Fig. 2), 1-year survival was 69% (95% CI: 62–76) and 2-year survival was 44% (95% CI: 37–51). Median PFS was 8.4 months (95% CI: 7.6–9.2), 1 and 2 year PFS was 35% (95% CI: 28–42) and 18% (95% CI: 12–24), respectively. According to univariate analysis, median OS was superior for PNET as compared with non-PNET (29 vs 20 months, HR = 0.65, 95% CI: 0.48–0.89; $P=0.006$), but this difference was not significant when adjusted for other factors (Table 4). In multivariable analysis, both age greater than 58 years and a poor PS were associated with a worse survival. Increasing grade according to Ki-67 was also associated with a significantly higher risk of death, with grade 3 patients having a 2.6-fold increased risk of death compared with patients with grade 1 tumours. The median survival for patients with ENETS grade G1, G2 and G3 was 29, 30 and 13 months, respectively.

Discussion

It is generally accepted that high-grade, poorly differentiated NET should be treated with platinum-based chemotherapy and that PNET are relatively sensitive to multi-agent streptozocin-based chemotherapy (Eriksson *et al.* 2009, Ramage *et al.* 2012). However, the role of chemotherapy for non-PNET has not been fully defined and, with the approval of sunitinib and everolimus for PNET, the position of chemotherapy in the therapeutic algorithm for PNET also needs to be clarified. The proliferation marker Ki-67 has been proposed as a means of selecting patients for chemotherapy and some evidence that response rate increases with increasing grade has been reported previously (Turner *et al.* 2010, Sorbye *et al.* 2013). Here, we sought to establish the predictive value to Ki-67% and define a meaningful threshold to aid patient selection. Ki-67 IHC staining was performed on FFPE samples taken from a range of sites including both primary and metastatic tumours at the time of diagnosis. The interval between the diagnostic biopsy and start of chemotherapy was variable and it is possible that some tumours may have migrated to a higher grade. However, in the absence of repeat biopsy before each line of therapy, the reliance on historic biopsies remains a limitation for such studies.

The patient population was evenly split between PNET and non-PNET and overall, 46% had G1 or G2

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tumours. The ratio of G3 to G1 tumours was higher in the non-PNET (5:1) compared with the PNET group (3:1). We found an association between response and Ki-67 grade, which ranged from 6 to 43% as grade increased from G1 to G3 according to the ENETS definition. However, we were unable to define a clinically useful threshold for Ki-67 index which would permit selection of patients for chemotherapy. Applying a Ki-67 of 35%, which gave the highest likelihood ratio, would deny 70% patients chemotherapy despite a response rate of 21% in this group. The ROC curve analysis confirmed the poor sensitivity and specificity of Ki-67 to predict response. Even among the 18 patients with a Ki-67 of ≤2%, there was one responder. Interestingly, we demonstrated that the response rate in non-PNET was lower at 22% compared with 39% despite the tendency to a higher grade in the non-PNET, and this is consistent with previous findings (Weatherstone & Meyer 2012). While we have shown that increasing Ki-67 grade is associated with a higher response rate to chemotherapy, we have also confirmed previous reports that it is a negative prognostic factor for survival with G3 grade tumours associated with a three-fold increased risk of death compared with G1 grade tumours after correcting for other variables (Rindi *et al.* 2006, 2007, Khan *et al.* 2013).

Although there has been no comparable study across the grade spectrum, our data concord with those of Sorbye and coworkers who looked exclusively at G3 tumours defined by Ki-67 >20 (Sorbye *et al.* 2013). In that study, response rate also rose as Ki-67 grade increased, while overall survival decreased. ROC curve analysis again suggested low sensitivity and specificity, but a cut-off of 55% was defined to distinguish those with a high chance of response (42%) vs low (15%). However, those with Ki-67 of 41–50% had a response rate of 21% suggesting that Ki-67 alone cannot be used to select patients. In contrast, a recent single centre study evaluating the combination of streptozocin and fluorouracil in patients with PNETs, reported a lower response rate for patients with a Ki-67 index of ≥15% at 25% compared with 46% for those with a Ki-67 <15% (Dilz *et al.* 2015). However, in this study, only 6% had a Ki-67 of >20%, and therefore the higher grade tumours were poorly represented in this patient sample. Additionally, in our series and that of Sorbye and coworkers, the majority of patients received platinum-based chemotherapy and Ki-67 may be a better predictor of response for this agent.

The expression of the DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) has also been proposed as a potential marker of response to alkylating agents such as temozolomide which is increasingly used for NET with response rates as high as 70% reported in PNET (Strosberg *et al.* 2011). However, the data for MGMT expression or promoter methylation in NET are conflicting. MGMT deficiency, as assessed by immunohistochemistry, ranges from 24 to 57% in pNET, with no cases of MGMT deficiency in GI-NET documented to date (Kulke *et al.* 2009, Gilbert *et al.* 2013, Liu *et al.* 2016). Kulke and coworkers reported an association between low MGMT expression and response in a cohort of 21 patients treated with TMZ-based chemotherapy (Kulke *et al.* 2009), and a more recent study of 53 patients treated with at least one alkylating agent demonstrated that patients with MGMT promotor methylation had a higher response rate than those without (Walter *et al.* 2015). Interestingly, Schmitt and coworkers reported no correlation between MGMT promotor methylation and MGMT expression in a large series of pNETS, and found that only promotor methylation was predictive of response to TMZ (Schmitt *et al.* 2014). By contrast, in a study of poorly differentiated NEC treated with temozolomide-based therapy, a response rate of 33% was reported despite only one of the 25 treated patients having MGMT promoter methylation (Welin *et al.* 2011). In the absence of a standardised assay applied to a large prospective series, it is not yet clear that MGMT expression or promotor methylation can be used to select patients.

In summary, we have demonstrated that increasing Ki-67 index is associated with increased response rates to chemotherapy, but cannot be used alone to select patients for this therapy. It may, however, provide a useful guide to inform patients of their likely chance of response and allow them to make informed choices about therapy. Achieving a response remains an important endpoint for patients who are symptomatic from tumour burden. Improved methods of patient stratification are therefore required in neuroendocrine tumours in order to provide benefit for those who will respond and spare toxicity in those who will not. There is considerable interest in the predictive role of circulating biomarkers, including NET-specific multi-gene transcripts (NET-test) (Modlin *et al.* 2014), cfDNA and CTCs (Khan *et al.* 2016), but further work is required to prospectively validate these markers before they can be used to select patients for chemotherapy (Modlin *et al.* 2014).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The guarantor of this article is Prof. Tim Meyer. Alexa Childs, Angela Lamarca, Doraid Alrifai, Roopinder Gillmore, Astrid Mayer, Christina Thirlwell, Debashis Sarker, Juan W Valle and Tim Meyer collected data. Alexa Childs, Tu Vinh Luong, Jennifer Watkins, Phyllis Nsiah-Sarbeng and Tim Meyer analysed data. Tim Meyer designed the research study. Alexa Childs and Tim Meyer wrote the paper. Amy Kirkwood and Julien Edeline performed statistical analysis. All authors reviewed and modified the final manuscript. All authors approved the final version of the manuscript.

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