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Clonal Diversity of *MYC* Amplification Evaluated by Fluorescent *In-Situ* Hybridisation and Digital Droplet Polymerase Chain Reaction in Oesophagogastric Cancer: Results from a Prospective Clinical Trial Screening Programme

Running title:

MYC amplification in oesophagogastric cancer

Authors:

Michael Davidson[1*], Lauren I Aronson[2*], Julie Howard-Reeves[1], Hanna Bryant[2], Rosalind J Cutts[2], Sanna Hulkki-Wilson[1], Kyriakos Kouvelakis[1], Eftheleria Kalaitzaki[1], David Watkins[1], Naureen Starling[1], Sheela Rao[1], Marta Llorca Cardenosa[2], Ruwaida Begum[1], Isma Rana[1], Retchel Lazaro-Alcausi[1], Monica Terlizzo[1], Andrew Wotherspoon[1], Gina Brown[1], John Swansbury[1], Christopher J Lord[2,3], David Cunningham[1], Ian Chau[1] and Irene Y Chong[1,2]

1. Royal Marsden NHS Foundation Trust, Downs Road, Surrey, SM2 5PT, UK

2. Institute of Cancer Research, 237 Fulham Road, London, SW3 6JB, UK

3. The Breast Cancer Now Toby Robins Research Centre, 237 Fulham Road, London, SW3 6JB, UK

* These authors contributed equally to the work

Co-corresponding authors:

Dr Irene Y Chong PhD MRCP FRCR

T +44 2071535138; E irene.chong@icr.ac.uk

Dr Ian Chau MD MRCP

T +44 2086613582; E ian.chau@rmh.nhs.uk

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Abstract

Introduction

The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancers. We report screening data from the i*MYC* trial, an ongoing phase II study assessing ibrutinib monotherapy in advanced pre-treated *MYC* and/or *HER2* amplified oesophagogastric cancer, representing the first attempt to prospectively identify *MYC* amplifications in this tumour type for the purposes of therapeutic targeting.

Methods

Screening utilising a fluorescent *in-situ* hybridisation (FISH) assay for assessment of tumour *MYC* amplification has been instituted. An experimental digital droplet polymerase chain reaction (ddPCR) assay to assess *MYC* amplification in both tumour and tumour-derived circulating (ct)DNA has been developed and investigated.

Results

135 archival tumour specimens have undergone successful FISH analysis with 23% displaying evidence of *MYC* amplification. Inter-tumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11- 94%). Intra-tumoural clonal diversity of *MYC* amplification was also observed, with a significant degree of variance in amplification ratios (bartlett test for equal variance $p<0.001$), and an association between greater variance in *MYC* amplification and improved outcome with prior first-line chemotherapy. ddPCR was most accurate in quantifying *MYC* amplification in tumour-derived DNA from cases with a high proportion (>70%) of amplified cells within the tumour specimen, but was not reliable in samples containing a low proportion of amplified cells or in ctDNA.

Conclusions

Our results illustrate the utility of FISH to assess *MYC* amplification prospectively for a biomarker-selected trial by providing reliable and reproducible results in real-time, with a high degree of heterogeneity of *MYC* amplification observed. We show that ddPCR can potentially detect high-level *MYC* amplifications in tumour tissue.

Key words

Oesophagogastric cancer, *MYC*, clonal diversity, prospective screening, FISH, digital PCR

Highlights

- First report of prospective screening for *MYC* amplification in OG cancer.
- *MYC* amplifications seen in 31/135 (23%) cases, with significant heterogeneity observed.
- Experimental ddPCR assay able to detect high-level tumoural *MYC* amplifications.
- Assessing *MYC* amplification for purposes of a biomarker-selected trial is feasible.

Introduction

The prognosis for patients with advanced oesophagogastric (OG) cancer is poor, with a median overall survival (OS) of less than 12 months in the majority of clinical trials [1]. There is utility for the targeted agents trastuzumab and ramucirumab in *HER2* positive first-line and unselected second-line settings respectively [2–4]. Further studies evaluating targeted agents inhibiting *EGFR*, *MET*, *PI3K/mTOR* and *PARP* have been disappointing, all failing to improve overall survival [5–9]. Therefore, effective biomarker-driven treatment approaches are urgently required to address this area of unmet clinical need.

The *MYC* proto-oncogene is among the most commonly dysregulated genes in human cancer, controlling the transcription of genes involved in multiple oncogenic pathways [10] [11]. Effective methods for targeting *MYC* represent an attractive potential therapeutic strategy. As it is a transcription factor that lacks enzymatic activity, *MYC* cannot be directly targeted by small molecule inhibitors or antibody-based therapies [12]. Strategies based on the concept of synthetic lethality, whereby disruption of two or more genes in combination result in a deleterious phenotype, have been investigated to indirectly target tumours displaying *MYC* over-activity [13, 14]. Using functional genomic screening of oesophageal cell lines, we previously observed selective decreased cell viability following silencing of Bruton's Tyrosine Kinase (*BTK*) in *MYC*-amplified cell lines. This was further validated using ibrutinib, a clinical BTK inhibitor that is also known to target *HER2* [15, 16]. In *MYC* amplified cells, ibrutinib down-regulated levels of MYC protein and downstream effectors, and elicited G1 cell cycle arrest and apoptosis. BTK signalling occurs partly via the canonical RAS-RAF-MEK-ERK pathway. ERK is a known mediator of MYC phosphorylation, and a putative mechanism of action for *MYC* and *BTK* interaction was identified through observation of BTK-dependent, ERK-mediated, MYC phosphorylation [16].

As a result of our pre-clinical observations, we initiated i*MYC* (NCT NCT02884453), an ongoing phase II non-randomised study to assess the efficacy of ibrutinib monotherapy in advanced pre-treated OG cancer that represents the first attempt at targeting *MYC* amplification clinically in this tumour type [17]. As there are no established definitions of *MYC* 'positivity' in solid organ tumours, previous studies investigating *MYC* amplification have used fluorescent *in-situ* hybridisation (FISH)-based techniques which defined positivity based upon established cut-offs derived from *HER2* FISH testing [18]. In parallel, we also developed a novel digital droplet polymerase chain reaction (ddPCR) assay to assess *MYC* amplification in both tissue and tumour-derived circulating (ct)DNA.

Here we give the first report of the frequency, pattern and heterogeneity of *MYC* amplification in OG cancer from a prospective screening study with clinical outcome correlation. We describe the development of a ddPCR assay using two independent reference probes, and assess the correlation of *MYC* amplification detected by FISH and ddPCR in both primary tumour tissue and ctDNA samples.

Materials and methods

Patient samples

Patients treated at the Royal Marsden Hospital, UK with OG cancer of either squamous cell carcinoma (SCC) or adenocarcinoma (AC) histological subtype being considered for or undergoing systemic anti-cancer therapy were eligible for screening. Informed written consent was obtained from all patients within the context of the i*MYC* trial (NCT02884453). At the time of consent, an archival diagnostic formalin-fixed paraffin-embedded (FFPE) tumour sample was obtained for analysis and blood samples were collected in Streck Cell-Free DNA blood collection tubes.

DNA extraction

FFPE tissue was cut into 5-8 micron sections and macrodissected to ensure >70% tumour content. DNA extraction was performed with the QIAamp DNA FFPE tissue kit (Qiagen) as per manufacturer's instructions. ctDNA was isolated from 4-5mL of plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) as per manufacturer's instructions. Tumour and ctDNA was quantified using the Qubit dsDNA HS assay (Thermo Fisher Scientific).

Fluorescent in-situ-hybridisation

A dual probe FISH assay was developed and validated to diagnostic quality for the purposes of the study. Screening was performed using a combination of a reference probe mapping to the centromere of chromosome 8 (CEP8) and a *MYC* probe mapping to chromosome 8q24, covering the entire coding region of the *MYC* gene from exons 1-3. Dual-label FISH was performed on each tissue section using standard techniques and results were reported in a standardised manner, recording both the range and modal ratio of CEP8 and *MYC* signals and, in the case of amplification, the proportion of cells displaying an amplified signal. The results were deemed to be amplified if *MYC*:CEP8 ratio ≥ 2.5.

Digital droplet PCR

A challenge in using ddPCR for the detection of gene amplifications is the reliable quantification of changes in the abundances of germline reference loci [19]. Suitable reference genes were identified through analysis of publically available copy number data to calculate copy number ratios of *MYC* versus all possible chromosome 8 reference genes in *MYC* amplified and non-amplified cancers. For the final analysis, two reference genes were chosen: a chromosome 8 reference gene, *CEBPD*, was selected on the basis of high sensitivity for *MYC* amplification and a chromosome 14 reference gene, *RPPH1*, frequently used in assessment of copy number variation. Data were analysed with respect to each control gene individually and the average ratio of *MYC*:*RPPH1* and *MYC*:*CEBPD* reported for each sample (see supplementary material S1 for additional description of ddPCR assay development and methodology).

Statistical analysis

Statistical analysis for ddPCR reference probes was carried out using the R statistical package (available at: http://www.R-project.org); all further statistical analysis was carried out using PRISM version 7 (available at: https://www.graphpad.com/scientific-software/prism). Categorical variables were compared using χ^2 test; continuous variables by Mann-Whitney U-test; progression-free survival (PFS) and OS by Kaplan–Meier method. Significance level for statistical tests was set at 0.05.

Results

Prospective patient cohort

Between July 2016 and January 2018, 162 patients were consented for prospective screening as part of the *iMYC* study. Eighty six percent (139/162) of cases were of AC histological subtype and 92% (149/162) had evidence of distant metastases at the time of enrolment. Of these, 88% of patients had received platinum-based first line chemotherapy treatment (table 1).

Evaluation of MYC amplification by FISH analysis of primary tumour FFPE samples

Samples were categorized as: 'diploid, copy number neutral' (displaying 2 *MYC* and 2 CEP8 signals per cell) (figure 1A), 'polysomatic' (additional signals of both *MYC* and CEP8) (figure 1B), '*MYC* amplified' (increased ratio of *MYC* to CEP8 signals) (figure 1C) +/- polysomy. Where a range of signal patterns was seen, the most prevalent (modal) pattern within the sample was recorded. Of 135/162 (85%) successfully analysed samples, a 'diploid, copy number neutral' signal pattern was seen in 24 (18%) cases (figure 1D). The most commonly observed pattern was polysomy without *MYC* amplification, seen in 80 (59%) cases. Amplification with no evidence of polysomy was observed in 16 (12%) samples and amplification with polysomy was seen in 15 (11%) samples. Inter-tumour heterogeneity was observed, with the percentage of cancer cells harbouring *MYC* amplification ranging widely between samples (median 51%, range 11-94%) (figure 2A). Intra-tumoural diversity as manifested by the range of *MYC* amplification present within each specimen, was observed with 22/31 (71%) amplified samples showing a range of amplification ratios present (figure 2B-C) and a significant variance in amplification ratios (bartlett test for equal variance $p<0.001$).

Clinical correlates of MYC amplification

We went on to assess association of *MYC* amplification with further histopathological features and response to prior systemic therapy. We observed that, although *MYC* amplification was identified in a higher percentage of SCC histological than AC subtypes the difference was not statistically significant (33 vs 21%; $p=0.22$ χ^2). We found no difference in *HER2* status, tumour differentiation or presence of signet cells between *MYC* amplified and non-amplified tumours (table S1). When considering the potential influence of *MYC* amplification on clinical outcome in patients with advanced disease treated with any first line systemic therapy (n=125), we found no significant difference in response rates (68 vs 55%; $p=0.22$ χ^2), median PFS (22.9 vs 22.1 weeks; $p=0.55$ log-rank; figure 3A) or OS (61.6 vs 63.3 weeks; $p=0.13$ log-rank; figure 3B) between *MYC* amplified and non-amplified tumours. Given the differences within the screening population in terms of first line treatment received, we then selected the patient group who had received a standard chemotherapy combination comprising a fluoropyrimidine and platinum agent with or without the addition of an anthracycline (n=84). For these patients, there was a trend towards improved overall response rates in the *MYC*-amplified cohort (64% vs 45%, $p=0.09$ χ^2) but amplification status again did not affect median PFS or OS. In an exploratory analysis, we assessed the impact of degree of clonal diversity in *MYC* amplification on survival outcome by undertaking tertile analysis based on the range of *MYC:CEP8* ratios observed within the amplified tumour specimens. We observed that tumour samples with the highest variance in *MYC* amplification were associated with a significantly longer PFS

following first line treatment (34.1 versus 18.7 weeks; log-rank *p*=0.0182; figure 3C) but no difference in OS was seen (68.7 vs 50.7 weeks; *p*=0.24 log-rank; figure 3D). The proportion of amplified cells within each tumour sample did not appear to influence survival.

MYC amplification assessment by ddPCR in patient samples from the iMYC trial

We then analysed patient samples retrieved as part of the i*MYC* study and, as of October 2018, 105 archival FFPE tumour samples have undergone DNA extraction. Of these, ddPCR analysis was successful in 98 (93%) (figure 4A). When comparing *MYC* amplification status as assessed by FISH and ddPCR, we observed a higher median tumour ddPCR ratio in *MYC* amplified as compared to non-amplified tumours (1.417 vs 1.246; *p*=0.017 Mann-Whitney U; figure 4B). However, the ddPCR ratios detected in OG tumours were lower than expected compared with cell line data (figure S3C), highlighting the difference between cell line and primary samples. Considering that we had observed significant inter- and intra-tumoural heterogeneity of *MYC* amplification, we hypothesised that ddPCR would have greater sensitivity in more highly-amplified cases. To address this we undertook a tertile analysis based on the proportion of amplified cells within the sample. Consistent with our hypothesis, we observed no significant difference in ddPCR ratio between amplified samples containing greater or less than 35% *MYC* amplified cells (1.432 vs 1.387; *p*=0.3111 Mann-Whitney U; figure 4C). In contrast, for samples containing greater or less than 70% amplified cells, the ddPCR ratio was found to be significantly higher in these more amplified samples (2.512 vs 1.396; *p*=0.0008 Mann-Whitney U; figure 4D), with a receiver operator area under the curve of 0.8958 (95% CI 0.7026-1.089; *p*=0.0015). Using a ddPCR cut-off ratio of 2.0 resulted in a sensitivity of 87.5% and specificity of 100% in identifying these highly (>70% cells) amplified cases.

ddPCR analysis using ctDNA was possible in 75/127 (59%) plasma samples, as the remainder had insufficient DNA for analysis (figure 4A). We observed no difference in plasma ddPCR ratios between *MYC* amplified and non-amplified tumours (1.547 vs 1.450; *p*=0.5705 Mann-Whitney U), and the percentage of cells harbouring *MYC* amplification in the primary tumour did not influence the ddPCR ratio.

Discussion

Based on pre-clinical work demonstrating a synthetically-lethal gene interaction between *BTK* and *MYC* [16], we have prospectively assessed *MYC* amplification for the first time in this tumour type for the purposes of therapeutic targeting with the BTK-inhibitor ibrutinib within a biomarker-selected clinical trial. Our screening results illustrate the utility of cytogenetic

analysis by FISH to assess *MYC* amplification in this context by providing reliable and reproducible results in real-time. We found *MYC* amplification in OG tumours assessed by FISH to be a relatively common event, occurring in 33% of SCC and 21% of AC tumours respectively. This is consistent with *MYC* amplification frequencies reported in a recent analysis of oesophageal AC and SCC undertaken by the Cancer Genome Atlas Research Network [20]. We observed a high degree of heterogeneity in amplification patterns, which has previously been described in a retrospective study in this tumour type [21]. Intra-tumoural heterogeneity in OG cancer, comprising both spatial heterogeneity and temporal heterogeneity along progression from primary to recurrent or advanced disease, is increasingly recognised [22], and heterogeneity of expression of *ERBB2* and *FGFR* genes have been associated with differential responses to targeted therapies in gastric cancer [23, 24]. Whether the heterogeneity of *MYC* amplification will impact upon the efficacy of its targeting within our current ongoing clinical trial remains to be seen however, this will be an important consideration in future efforts to target *MYC* from a clinical perspective.

We observed a trend towards improved response to first line platinum/ fluoropyrimidine-based chemotherapy in *MYC*-amplified tumours, consistent with previously described associations between *MYC* amplification and favourable chemotherapy response in breast and ovarian cancer [25–27]. Although *MYC* status did not influence survival outcomes overall, we identified an association with improved PFS in cases with a higher variance in observed amplification ranges within the specimen. *MYC* amplification has been associated with a tumour-hypoxic molecular signature indicating increased underlying genomic instability [11]. Genomic instability has also been correlated with improved outcomes to platinum-based chemotherapy [28] and it is possible that clonal diversity of *MYC* amplification may be a surrogate for this. However further work is necessary to clarify the role of *MYC* in mediating platinum response in this disease, and validation of our findings in a larger independent dataset will be required.

Given the heterogeneity seen, identifying amplifications from pooled DNA as compared to single cell-based analysis such as FISH is likely to be challenging. Our novel ddPCR assay was developed based upon robust identification of suitable reference genes. Although a statistically significant difference in tumour ddPCR ratio was seen between *MYC* amplified and non-amplified tumours as assessed by FISH, the absolute difference was small. However ddPCR was able to identify more highly amplified tumour samples (containing >70% amplified cells). Thus the ability of ddPCR to detect *MYC* amplifications may be limited to those tumour samples displaying a homogenous high-level pattern of *MYC* amplification only. Its application to ctDNA was limited by the relatively small numbers of samples where adequate DNA could be extracted and successfully analysed. The high failure rate encountered is likely to be due,

in part, to the nature of the i*MYC* pre-screening cohort as patients could have blood taken at any time before, during or after a line of treatment. Effective systemic treatment can potentially reduce absolute ctDNA levels [29], thus the yield of extracted ctDNA from plasma was potentially influenced by the clinical context at the time point when the blood was taken. The accuracy of ctDNA ddPCR in detecting genomic amplifications is known to be lower than tumour ddPCR [30], and the high heterogeneity of *MYC* amplification may have contributed to the lower concordance seen [21]. Furthermore, *MYC* amplification has been associated with both aneuploidy and increased intra-tumoural heterogeneity in other solid tumours, and may be an acquired event in tumour evolution from primary to metastatic disease, potentially affecting equivalence of results between primary tumour specimens and ctDNA from blood samples taken at differing timepoints of disease progression [31, 32].

As research into effective *MYC*-targeting treatment continues robust and reproducible methods of biomarker detection will be necessary, and we show that prospective screening for *MYC* amplification in OG cancer for the purposes of therapeutic targeting is feasible. The FISH assay used has revealed *MYC* amplification to be a common event in this tumour type, with a high degree of heterogeneity of *MYC* amplification patterns observed. We show that ddPCR can potentially be used to detect *MYC* amplifications in tumour samples with a high proportion of amplified cells, however further work is necessary to optimise this technique in ctDNA. Although a trend towards improved chemotherapy response rates in *MYC*-amplified patients was noted, no clear association between *MYC* status and survival was seen. The clinical implications of *MYC* alterations in OG cancer are therefore likely to lie in the potential to harness *MYC* as a therapeutic target, and the clinical efficacy results of our biomarker-selected trial are awaited.

Conflict of interest:

NS: Research funding: AstraZeneca, BMS, Merck. Honoraria: AstraZeneca.
DC: Research funding: Amgen, AstraZeneca, Bayer, Celgene, Merck-Serono, Medimmune, Merrimack, Novartis, Roche, Sanofi.
IC: Advisory Board: Eli-Lilly, Bristol Meyers Squibb, MSD, Bayer, Roche, Merck-Serono, Five Prime Therapeutics, Astra-Zeneca, Oncologie International, Pierre Fabre
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Supplementary material S1:

Development and methodology of ddPCR assay

Figure legends:

Figure 1. Patterns of *MYC* amplification in primary oesophagogastric tumour samples detected by FISH (red= *MYC*; green= CEP8). (A) Normal diploid pattern (2 *MYC* and 2 CEP8 signals per cell); (B) Polysomic pattern (additional signals of both *MYC* and CEP8); (C) *MYC* amplified pattern (increased ratio of *MYC* to CEP8 signals); (D) Flow diagram indicating the results of *MYC* FISH assessment in i*MYC* trial samples, separated based on histology.

Figure 2. Inter- and intra- tumoural heterogeneity of *MYC* amplification. (A) bar chart illustrating proportion of cells displaying *MYC* amplification within each amplified specimen; (B) line chart demonstrating range of *MYC* amplification ratios seen within each amplified specimen; (C) individual sample showing intra-tumoural heterogeneity of *MYC* amplification patterns; green arrow= normal diploid; yellow arrow= polysomatic; red arrow= *MYC* amplified.

Figure 3. Influence of *MYC* amplification status on survival. (A) Progression free survival on first line systemic treatment for *MYC* amplified versus non-amplified tumours by FISH (22.9 vs 22.1 weeks; p=0.55 log-rank); (B) Overall survival for *MYC* amplified versus non-amplified tumours by FISH (61.6 vs 63.3 weeks; p=0.13 log-rank); (C) Progression free survival for high variance (defined as top tertile of tumours by range of *MYC* amplification observed within specimen) versus low/ medium variance (low and mid tertile tumours by range of *MYC* amplification observed within specimen) tumours (34.1 vs 18.7 weeks; p=0.0182 log-rank); (D) Overall survival of high variance versus low/ medium variance *MYC* amplified tumours (68.7 vs 50.7 weeks; p=0.24 log-rank).

Figure 4. *MYC* amplification assessment by ddPCR in patient samples from the i*MYC* trial (A) Flow diagram of tumour and ctDNA ddPCR sample analysis from patients entered into the i*MYC* trial; Scatter plots showing (B) significant difference in tumour ddPCR ratio between *MYC* amplified and non-amplified samples assessed by FISH (p=0.017 Mann Whitney U); (C) no difference in tumour ddPCR ratio between *MYC* amplified samples with< 35% and >35% amplified cells, respectively (p=0.3111 Mann Whitney U); (D) a significant difference in tumour ddPCR ratio between *MYC* amplified samples with <70% and >70% amplified cells, respectively (p=0.008 Mann Whitney U).

Supplementary figure 1. Identification of suitable reference genes for probes in ddPCR assay. (A) Workflow for identifying ddPCR reference probe; (B) Analysis of copy number data from

443 gastric and 186 oesophageal primary cancers. The ratio of *MYC* to each gene on chromosome 8 was calculated and this was compared in *MYC* amplified and non-amplified cancers by Student's t test. Arrows indicate the position of *CEBPD* and *MYC*. (C) The corresponding sensitivity was assessed for each gene along chromosome 8. (D) Comparison of *MYC*:*CEBPD* and *MYC*:*RPPH1* copy number ratios in *MYC* amplified (AMP) and non-amplified (NON-AMP) cancers from the same datasets.

Supplementary figure 2. Comparable results generated when ddPCR multiplex reactions read using the (A) FAX/VIC channel or (B) FAX/HEX channel.

Supplementary figure 3. Development of ddPCR assay; (A) Real-Time quantitative PCR demonstrates equal amplification from all three probes used in the ddPCR assay with the slope and reaction efficiency for each standard curve similar, indicative of unbiased amplification; (B) Correlation between cell line *MYC* copy number (CN) as determined by CCLE and *MYC* copy number as determine by ddPCR. Cell lines with known *MYC* amplifications are highlighted in red (Pearson r coefficient=0.799; two-tailed $p<0.0001$); (C) aCGH data used to classify oesophageal cell lines into either amplified (AMP) or non-amplified (Non-AMP) groups ($p=0.0732$, Mann Whitney U); (D) 2D plot of ddPCR data showing fluorescence from channel 1 (*MYC*) plotted against fluorescence from channel 2 (reference gene) for each droplet, with the drops clustering into groups as shown. Data are displayed for a *MYC* amplified ctDNA sample (ratio= 11.96; upper plot) and *MYC* non-amplified ctDNA sample (ratio= 1.34; lower plot).

Table legends:

Table 1. Patient characteristics of i*MYC* pre-screening population.

Table S1. Contingency tables showing distribution of clinico-pathological variables between *MYC* amplified and non-amplified samples.

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Statement of author contributions:

Study concept: MD, IYC, IC
Study design: MD, IYC, IC
Data acquisition: MD, RLA, RB, DC, DW, NS, SR, IC
Quality control of data and algorithms: MD, IYC, KK, EK
Data analysis and interpretation: MD, IYC, LA, HB, JHR, RC, SHK, KK, EK, MLC, MT, AW, GB, JS, CJL
Statistical analysis: MD, RC, KK, EK
Manuscript preparation: MD, LA, IYC
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All authors were involved in preparation of the manuscript and had final approval of the submitted version.