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Targeting EGFR pathway in metastatic colorectal cancer- tumour heterogeniety and convergent evolution

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ABSTRACT

Despite significant progress in management of metastatic colorectal cancer (mCRC) pertaining to better screening procedures and amelioration of the therapeutic armamentarium with targeted therapies, prognosis remains poor. Targeting epidermal growth factor receptor (EGFR) has been of particular interest owing to favourable efficacy benefits demonstrated by monoclonal antibodies (cetuximab and panitumumab) in various clinical settings and development of predictive biomarkers informing treatment decisions respectively. In spite of optimal patient selection based on *RAS* mutation status, primary and secondary resistance to monoclonal antibodies is higher than desired.

Further research into predictive biomarkers is therefore essential, but has, to date, been conducted with considerable limitations. Whilst molecular heterogeneity has been demonstrated by several studies in mCRC, for incomprehensible reasons, multiple resistant genetic alterations that emerge under the selective pressure of EGFR-targeted therapies are somehow able to influence the biological and clinical behaviour of cancer cells, despite being detectable at extremely low frequencies. Intriguingly, these subclonal events largely seem to converge on RAS/RAF/MAPK pathway in patients treated with EGFR-targeted monoclonal antibodies. This review describes the clinical and biological evolution and development of EGFR targeted therapies in mCRC, the challenges in the presence of molecular complexities, the role of cell free (cf)-DNA and future strategies that could lead to further optimal discovery of clinically meaningful biomarkers and application of precision medicine.

1. Introduction

Colorectal cancer (CRC) is one of the commonest cancers in the world and is associated with a high morbidity and mortality (DeSantis et al., 2014; Ferlay et al., 2013; Khan et al., 2015; Siegel et al., 2013) and patients with metastatic CRC (mCRC) have particularly dismal outcomes(Lieberman, 2012). Targeting epidermal growth factor receptor (EGFR) has been a successful strategy in mCRC in the absence of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) or neuroblastoma RAS viral homolog (*NRAS*) mutations; however, despite optimal patient selection, only 35–45% and 10–20% patients respond to first-line multidrug chemotherapy combination and anti-EGFR monotherapy respectively(Lee and Kopetz, 2015). Even those patients who derive clinical benefit develop secondary resistance inevitably. One of the key reasons for this lack of therapeutic progress in mCRC is intrinsic heterogeneity which is better studied but less well understood in the metastatic setting (Misale et al., 2014). In patients treated with anti-EGFR therapies, molecular heterogeneity is known to play a pivotal role in acquired compared to *de novo* resistance. Small retrospective studies have shown that under the selective pressure of anti-EGFR therapies, subclonal events can influence the clinical outcomes (Misale et al., 2014); more recently, our group demonstrated that low frequency RAS pathway alterations can influence clinical outcomes in patients treated with anti-EGFR therapies within a prospective clinical trial(Khan et al., 2018). However, the mechanisms through which a small subclone confers resistance in the presence of larger wild type (WT) allele are incompletely understood. In this review, we will discuss the evolution of EGFR therapies in mCRC, challenges in selection of patients for such therapies and future trial designs that can help us better understanding the disease biology and overcome resistance mechanisms.

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2. EGFR targeting monoclonal antibodies in colorectal cancer

Cetuximab and pantitumumab are monoclonal antibodies (mAbs) which competitively bind to the extracellular domain of EGFR and block phosphorylation and activation of the receptor tyrosine kinases resulting in inhibition of cell growth, induction of apoptosis and decrease in matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) production (Khan, 2015; Mendelsohn and Baselga, 2006). Although their mechanism of action is incompletely understood it is believed to be through a combination of inhibition of dimerisation of EGFR with other erythroblastosis oncogene B (ErbB) family members leading to blocking of tyrosine kinase domain phosphorylation and pathway inactivation. Moreover, cetuximab may induce antibody dependent cell cytotoxicity (ADCC) thus promoting immunogenic cell death and immune related effects of the therapy(Kurai et al., 2007; Lo Nigro et al., 2016; Pozzi et al., 2016; Saltz et al., 2004; Trivedi et al., 2016; Trotta et al., 2016).

EGFR is a glycoprotein that belongs to the ErbB family member receptor tyrosine kinase (RTK), straddling the membrane with an extracellular ligand-binding domain and an intracellular tyrosine-kinase domain(Scaltriti and Baselga, 2006).

EGFR remains in the state of inhibition in the absence of specific ligands such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), epiregulin (EREG), betacellulin, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), epigen, heregulin, and neuregulins 1-4(Yarden and Sliwkowski, 2001). The binding of cognate ligands to the extracellular domain induces homo or heterodimerisation with other ErbB RTKs, which triggers phosphorylation of the tyrosine kinase domain. This activates signal transduction through the RAS-RAF-MAPK pathway, which ultimately promotes tumour growth and progression(Hynes and Lane, 2005).

Signal transduction through EGFR also activates the neighbouring phosphatidylinositol-3-kinase (PI3K) - AKT signalling cascade, which is critical to cell survival, motility and invasion (Downward, 1998; Khan et al., 2013; Lemmon and Schlessinger, 1994), thus further promoting cancer survival and progression **(**Fig. 1**).**

3. Primary resistance to anti-EGFR monoclonal antibodies

3.1. Evolution of KRAS as a biomarker

Anti-EGFR mABs including cetuximab and panitumumab have demonstrated efficacy in mCRC both as monotherapy and in combination with chemotherapy (Jonker et al., 2007; Price et al., 2014; Van Cutsem et al., 2007). In search of biomarkers, EGFR was initially thought to be relevant in CRC because of two reasons: 1) up-regulation of the EGFR gene was found to be common (30–70%) event, (Messa et al., 1998) and its over-expression was associated with poorer survival in CRC patients (Mayer et al., 1993); and 2) in non-small cell lung cancer (NSCLC), a series of studies demonstrated efficacy of EGFR inhibitor therapy in patients with an activating mutation in EGFR genes (Siegelin and Borczuk, 2014). However, assessment of over-expression of EGFR by immunohistochemistry (IHC) methods did not show an association with response to anti-EGFR mABs (Chung et al., 2005; Cunningham et al., 2004; Saltz et al., 2004). Moreover, fluorescent in-situ hybridisation (FISH) assessment of EGFR gene copy number demonstrated positive predictive value for gefitinib in a proportion of NSCLC studies(Hirsch et al., 2006; Zhu et al., 2008), no clear evidence for the role of EGFR gene copy number as a predictive biomarker of response to anti-EGFR mABs in CRC exists(Barton et al., 2010).

Following failure of EGFR mutation or copy number changes as predictor of response to anti-EGFR mAbs in mCRC (Chung et al., 2005; Hecht et al., 2010); the next logical step was the examination of the downstream effectors of the EGFR-signalling pathway. The hypothesis was that mutations in the gene expressing the cell signal transducer *KRAS* may potentially leads to the expression of a constitutively active *KRAS* protein. As a result, there is stimulus-independent auto-phosphorylation of *KRAS* and activation of the EFGR signalling pathway downstream of *KRAS*, invoking resistance to mABs targeting the upstream EGFR receptor(Benvenuti et al., 2007). *KRAS* is a member of the RAS family of oncogenes (that also includes *HRAS* and *NRAS*) which encode guanosine triphosphate-binding proteins(Malumbres and Barbacid, 2003). There are a discrete number of mutations in *KRAS* which are readily detectable and cause constitutive activation of the

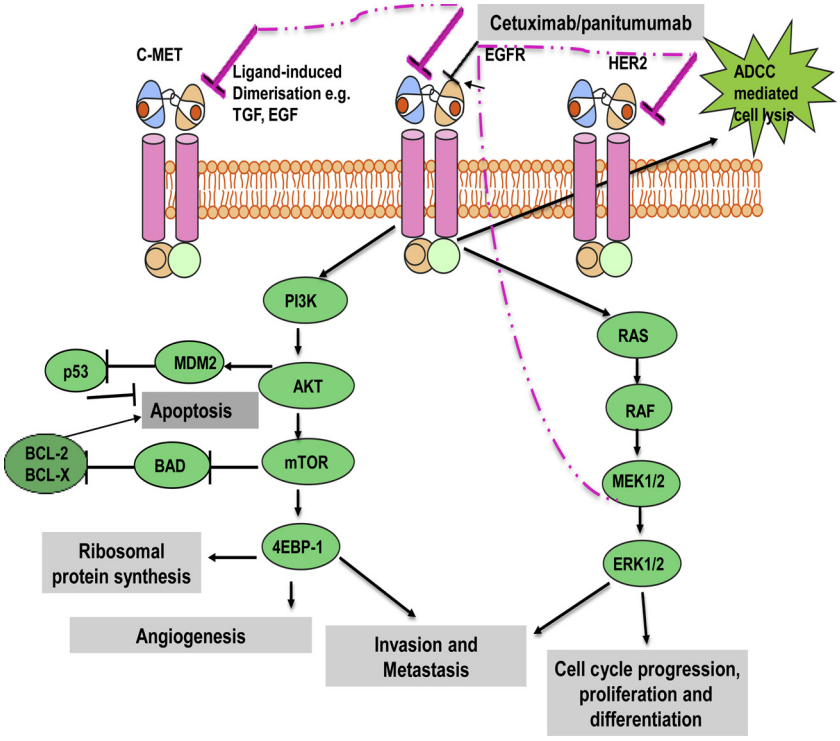

Fig. 1. Schematic diagram of cetuximab induced blockade of EGFR signal transduction pathway.
The epidermal growth factor receptor (EGFR) belongs to one of the four members of erbB family of receptor tyrosine kinases. It consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Binding of a ligand like TGF-α or EGF to EGFR causes receptor dimerisation. This leads to receptor autophosphorylation which results in activation of downstream pathways by signal-transduction cascade involved in cell proliferation and survival. The two distinct pathways include RAS-RAF-MEK-ERK pathway and the phosphoinositide 3-kinase-serine/threonine kinase (PI3K-AKT) pathway, which are further linked with apoptosis via MDM2/TP53 and BAD/BCL-2 pathways. Cetuximab/panitumumab blocks binding of ligands to EGFR, thereby inhibiting the receptor phosphorylation and downstream events. It also causes Antibody dependent cell cytotoxicity (ADCC) dependent cell lysis which contributes to its anti-cancer activities. Mammalian target of rapamycin (mTOR), 4E-binding protein (4EBP-1), MAPK, mitogen-activated protein kinase; TGF-α, transforming growth factor-α; TK, tyrosine kinase domain. Pink dotted lines represent potential strategies in overcoming resistance to anti-EGFR therapies.

Table 1
Clinical studies of Anti-EGFR therapy in mCRC.

First author	Number of patients	Treatment arms	ORR (%)		PFS (months)		OS (months)	
First line studies			ITT	*KRAS* WT	ITT	*KRAS* WT	ITT	*KRAS* WT
OPUS	337 (n = 179/315 *KRAS* codons 12,13 WT)	FOLFOX 4 FOLFOX 4 +cetuximab	36	34	7.2	7.2	18.3	18.5
			46	57	7.2	8.3	18.0	22.8
			p = 0.64	p = 0.0027	p = 0.617	p = 0.0064	p = 0.91	p = 0.39
CRYSTAL	1198 (n = 666/1063 *KRAS* codons 12, 13 WT)	FOLFIRI FOLFIRI + cetuximab	38.7	39.7	8	8.7	18.6	20.0
			46.9	57.3	8.9	9.9	19.1	23.5
			p = 0.04	p = < 0.001	p = 0.048	p = 0.0012	p = 0.31	P = 0.0093
COIN	1630 (n = 729/1316 *KRAS* codons 12, 13 and 61 WT)	FOLFOX or CAPOX FOLFOX or CAPOX + cetuximab	NR	57	NR	8.6	NR	17.9
			NR	64	NR	8.6	NR	17
				p = 0.049		p = 0.60		p = 0.67
NORDIC-VII	379 (n = 194/324 *KRAS* codons 12,13)	Bolus FLOX Bolus FLOX + cetuximab	41	47	7.9	8.7	20.4	22.0
			49	46	8.3	7.9	19.7	20.1
			p = 0.15	p = 0.87	p = 0.31	p = 0.66	p = 0.67	p = 0.48
PRIME	1198 (n = 666/1063 *KRAS* codons 12,13 WT)	FOLFOX FOLFOX + panitumumab	NR	48	NR	8.0	NR	19.4
				55		9.6		23.8
				p = 0.068		p = 0.02		p = 0.03
Second line or beyond								
CO.17	572 (n = 394/572 *KRAS* codons 12,13 WT)	Cetuximab BSC	8.0	13	1.9	3.7	6.1	9.5
			0	1	1.8	1.9	4.6	4.8
			p = 0.001	p < 0.0001	p < 0.001	p < 0.001	p = 0.005	p < 0.001
EPIC	1298	Irinotecan Irinotecan + panitumumab	4.2	NR	2.6	NR	10.7	NR
			16.4	NR	4.0	NR	10.0	NR
			p = < 0.0001	p = < 0.0001			p = 0.71	
Van Cutsem et al.	463 (n = 243/427 *KRAS* codons 12, 13 WT)	BSC BSC + cetuximab	0	0	1.6	1.6	NR	7.6
			10	17	1.8	2.8	NR	8.1
			p = < 0.0001		p = < 0.0001	p = < 0.0001	(HR = 1.0)	(HR = 0.67)
PICCOLO	1198 (n = 460/1198 *KRAS* codons 12,13 and 61 WT)	Irinotecan Irinotecan + panitumumab	N/A	12	N/A	4.7	N/A	10.9
			N/A	34	N/A	5.5	N/A	10.4
				p = < 0.0001		p = 0.015		p = 0.91
Study 20050181	1186 (n = 597/1083 *KRAS* codons 12, 13 WT)	FOLFIRI FOLFIRI + panitumumab	N/A	10	N/A	4.9	N/A	12.5
			N/A	36	N/A	6.7	N/A	14.5
				p = < 0.0001		p = 0.015		p = 0.37

BSC = best supportive care; CAPOX = capecitabine plus oxaliplatin; CRYSTAL = Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer; EGFR = epidermal growth factor receptor; FLOX = bolus 5-fluorouracil/leucovorin/oxaliplatin; EPIC = Erbitux Plus Irinotecan for Metastatic Colorectal Cancer; FOLFIRI = 5-fluorouracil/leucovorin/irinotecan; HR = hazard ratio; ITT = intention to treat population; KRAS = Kirsten rat sarcoma viral oncogene homolog; mCRC = metastatic colorectal cancer; N/A = not applicable; NR = not reported; OPUS = Oxaliplatin and Cetuximab in Fist-line Treatment of Metastatic Colorectal Cancer; OS = overall survival; ORR = overall response rate; PFS = progression free survival; PICCOLO = Panitumumab, Irinotecan, and Ciclosporin in Colorectal Cancer; PRIME = Panitumumab Randomised Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy; WT = wild type.

EGFR signalling pathway, and these are usually encoded on codons 12 and 13(Jimeno et al., 2009). The frequency of *KRAS* mutation in mCRC is approximately 40% (Amado et al., 2008; Karapetis et al., 2008; Maughan et al., 2011; Van Cutsem et al., 2009).

Retrospective analysis of archived clinical trial specimens demonstrated that the presence of a mutation in *KRAS* was a predictive marker of resistance to EGFR-targeted mABs. In patients with previously treated *KRAS* WT mCRC there was significant improvement in ORR (Amado et al., 2008; Karapetis et al., 2008) and PFS to both cetuximab or panitumumab when patients were segregated based on their *KRAS* mutation status (Amado et al., 2008; Lee and Kopetz, 2015). Likewise, the addition of anti-EGFR mAB to chemotherapy in first line treatment of *KRAS* WT mCRC consistently resulted in improvement of ORR (Bokemeyer et al., 2009; Douillard et al., 2010; Maughan et al., 2011; Van Cutsem et al., 2009, 2011) and most of these studies additionally demonstrated significant improvement in PFS in favour of the mAb containing arm (Bokemeyer et al., 2009; Douillard et al., 2010; Van Cutsem et al., 2009, 2011) (Table 1). However, only one of these studies showed a significantly improved OS of 3.5 months (23.5 months in the cetuximab-containing arm) (Van Cutsem et al., 2009, 2011) and others showed a non-significant trend towards improved OS (Lee and Kopetz, 2015). Panitumumab was also shown to have efficacy in second line treatment of *KRAS* WT mCRC, improving ORR from 10% to 35% and median PFS from 3.9 to 5.9 months when added to FOLFIRI compared to FOLFIRI alone (although again no significant improvement in OS was shown)(Peeters et al., 2010).

Contrary to expectation, the largest study investigating the EGFR mAB and chemotherapy combination in first-line treatment of mCRC (COIN) failed to confirm predictive association of *KRAS* mutation with improved PFS or OS with cetuximab (although a small improvement in ORR was demonstrated)(Maughan et al., 2011). The high incidence of gastrointestinal toxicity in the large proportion of patients receiving cetuximab combined with capecitabine and oxaliplatin, mandating dose reduction, is a possible reason for this result(Maughan et al.,

2011). Finally, NORDIC-VII trial also failed to demonstrate improvement in ORR, PFS or OS in patients treated with FLOX alone or in combination with cetuximab in first-line mCRC(Tveit et al., 2012). These results brought concerns about the efficacy of combining anti-EGFR mABs with oxaliplatin based regimens; however, the recent data from Cancer and Leukaemia Group B (CALGB)/Southwest Oncology group (SWOG) 80405 study demonstrated equivalent outcomes between patients treated with various chemotherapy backbones including oxaliplatin based regimens in combination with cetuximab or bevacizumab(Elez et al., 2015).

Overall, the evidence for *KRAS* mutational status as a biomarker of resistance to cetuximab and panitumumab was however considered sufficiently persuasive to prompt the European health authorities and the US FDA (Food and Drug Administration) to recommend restriction of panitumumab and cetuximab treatment in patients with mCRC to those with WT *KRAS* mCRC.

3.2. Extended RAS testing and its predictive significance as a biomarker

More recently it has been established that whole RAS gene confers a predictive role in response or resistance to anti-EGFR targeted therapies (Douillard et al., 2013; Peeters et al., 2013). Douillard et al. conducted extended RAS analysis of the available tissue from PRIME study (Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy). This study initially compared efficacy and safety of FOLFOX4 alone with FOLFOX4 along with panitumumab in chemo-naive patients with metastatic colorectal cancer. *KRAS* status was established by mutations in exon 2 at the time of initial analysis. Subsequently mutation analysis was extended to *KRAS* exon 3 (codon 61), and exon 4 (codons 117 and 146); *NRAS* exon 2 (codons 12 and 13), exon 3 (codon 61) and exon 4 (at codons 117 and 146); and *BRAF* exon 15 (codon V600). It was established that a total of 17% (108/620) patients without *KRAS* mutations in exon 2 had mutations in other *RAS* exons; the updated analysis of this subgroup showed a trend towards inferior PFS and OS in panitumumab-FOLFOX-4 group compared to FOLFOX alone, although the difference was not statistically significant. In patients with pan-*RAS* WT tumours, PFS of 10.1 versus 7.9 months ($p = 0.004$), and OS of 26.0 versus 20.2 months ($p = 0.04$) was observed in the panitumumab-FOLFOX-4 combination arm. Patients with no mutation in *KRAS* exon 2 but mutations in another *KRAS* or *NRAS* loci, a non-significant trend towards inferior PFS (7.3 vs. 8.0 months, $p = 0.33$) and OS (17.1 vs. 17.8 months, $p = 0.12$) was noted; however, with significant interaction test ($p = 0.04$ for PFS and $p = 0.01$ for OS). Following this, extended RAS testing was conducted in numbers of other previous studies and revealed that 15–26% of mCRC patients previously labelled as *KRAS* WT harboured mutations in other loci of *KRAS* or *NRAS* genes, which meant that they were likely to be resistant to anti-EGFR therapies (Table 2). FIRE-3 study that compared FOLFIRI plus cetuximab to FOLFIRI plus bevacizumab as first line treatment in patients with mCRC according to *KRAS* status, demonstrated that patients with pan *RAS*-WT tumours had a higher ORR (76% vs. 65.2%) and OS (33.1 m vs. 25.9 m) in the cetuximab group compared to the bevacizumab treated patients (Stintzing et al., 2012).

These data changed prescription guidelines so that only patients with pan-*RAS* WT tumour are now offered anti-EGFR therapy within the context of mCRC.

3.3. Other potential markers for primary resistance to EGFR mABs

Interrogation of the signalling cascade downstream of EGFR has also revealed other potential biomarkers of EGFR mAB efficacy in mCRC. *BRAF* is a protein immediately downstream of *RAS*, and it acts as one of its main effectors that need to be phosphorylated by *KRAS* for its activation. Mutations in the gene encoding *BRAF* occur with a frequency of approximately 8–10% in mCRC, and *BRAF* mutations are mutually exclusive with *KRAS* mutations (De Roock et al., 2010; Maughan et al., 2011). More than 90% of the mutations in *BRAF* associated with constitutive activation of EGFR signalling involves a single amino acid substitution (V600E)(Rajagopalan et al., 2002). This *BRAF* mutation has been associated with a very aggressive biology associated with detrimental clinical outcome (PFS and OS) (Di Nicolantonio et al., 2008; Laurent-Puig et al., 2009). The negative prognostic values of *BRAF* mutation was further confirmed by PRIME and PICCOLO (Panitumumab, Irinotecan, and Ciclosporin in Colorectal Cancer studies) (Douillard et al., 2013; Seymour et al., 2013) studies. Furthermore two meta-analyses confirmed the poor prognostic role of activating *BRAF* mutation along with lack of efficacy to EGFR inhibitors(Pietrantonio et al., 2015; Rowland et al., 2016); however, the aggressive phenotype along with significant negative prognostic significance makes the predictive value of this marker more difficult to interpret (Maughan et al., 2011; Tol et al., 2010).

The Phosphatidylinositol 3-kinase (PI3K)/AKT pathway is another EGFR-activated intracellular signalling channel which is essential to cell growth and survival (Cantley, 2002; Khan et al., 2013; Osaki et al., 2004). *PIK3CA* mutations are reported in 6–14% of mCRC patients (De Roock et al., 2010; Di Fiore et al., 2010). The results of studies investigating the predictive significance of the *PIK3CA* mutations are conflicting: some show an association with resistance to EGFR mAB treatment(De Roock et al., 2010; Perrone et al., 2009; Sartore-Bianchi et al., 2009), but others have failed to demonstrate this(Prenen et al., 2009; Tol et al., 2010), potentially due to small sample sizes. Additionally, only some of the *PIK3CA* mutations that have been studied may have predictive significance and need further validation.

Increased expression of the alternate EGFR ligands epiregulin (EREG) and ampiregulin (AREG) as detected by mRNA have also been associated with cetuximab sensitivity(Jacobs et al., 2009; Khambata-Ford et al., 2007). As both are ligands for the EGFR they have been investigated as potential predictive biomarkers of EGFR pathway inhibition (Jacobs et al., 2009; Jonker et al., 2014; Khambata-Ford et al., 2007). Seligmann et al. described the prognostic and predictive effects of AREG and EREG gene expression levels on outcomes in the PICCOLO study(Seligmann et al., 2016; Smyth et al., 2016). Overall, the data from this study may not prove to be practice changing but may have a valuable impact on patient selection if these data are validated in a bigger prospective cohort(Smyth et al., 2016).

Finally, more recent evidence points towards the impact of anatomy of tumour (i.e. right vs. left sided) on the clinical outcomes. The results of CALBG/SWOG 80405 study showed that mCRC arising from right or left colon were clinically different with statistically superior OS in patients with left sided colonic cancers. In a non pre-planned analysis, it was also demonstrated that PFS and OS were superior in cetuximab treated left sided colonic tumours (32.1 vs. 16.4 months, $p = < 0.0001$, HR = 1.97)(Venook et al., 2016). A multi-variate analysis considering other prognostic and predictive factors was required and indeed the pooled analysis of FIRE-3 and CRYSTAL studies showed that in the RAS WT populations, patients with left-sided tumors had a markedly better prognosis than those with right-sided tumors. Moreover, the authors concluded that first-line FOLFIRI plus cetuximab was clearly associated with significant benefit in patients with left-sided tumors (vs. FOLFIRI or FOLFIRI plus bevacizumab, respectively), whereas patients with right-sided tumors derived limited benefit (Tejpar et al., 2016).

4. Proposed mechanisms of acquired resistance

Efficacy of anti-EGFR mABs in mCRC is often short-lived, regardless of the chemotherapy backbone and/or line of treatment. Patients selected based on their *RAS* WT status, even in a best case scenario usually succumb to progressive disease within 12–18 months and 6–9 months of receiving first line combination chemotherapy and with single agent mABs in refractory settings respectively(Price et al., 2014; Van Cutsem et al., 2011).

The mechanisms for acquired resistance to anti-EGFR mABs in

Table 2
Retrospective extensive RAS testing results in previous studies of Anti-EGFR therapy in mCRC.

First author	Number of patients with extended RAS testing	Treatment arms	ORR (%)		PFS (months)		OS (months)	
First line studies			**KRAS WT**	***Extended RAS***	**KRAS WT**	***Extended RAS***	***KRAS* WT**	***Extended RAS***
PRIME	1198 (n = 108/620 with pan-RAS WT)	FOLFOX	48	NR	8.0	7.9	19.4	20.2
		FOLFOX + panitumumab	55	NR	9.6	10.1	23.8	26.0
			p = 0.068		p = 0.02	p = 0.004	p = 0.03	p = 0.04
OPUS	337 (n = 31/118 with pan-RAS WT)	FOLFOX 4 FOLFOX 4 +cetuximab	34	28.6	7.2	5.8	18.5	17.8
			57	57.9	8.3	12.0	22.8	19.8
			p=0.0027	p=0.008	p=0.0064	p=0.062	p = 0.39	p = 0.80
CRYSTAL	1198 (n = 63/430 with pan-RAS WT)	FOLFIRI FOLFIRI + cetuximab	39.7	38.6	8	8.4	20.0	20.2
			57.3	66.3	8.9	11.4	23.5	28.4
			p= < 0.001	p= < 0.0001	p = 0.048	p=0.0002	p = 0.0093	p = 0.0024
Second line or beyond								
Study 20050181	1186 (n = 107/597 with pan-RAS WT)	FOLFIRI	10	NR	4.9	4.6	12.5	13.9
		FOLFIRI + panitumumab	36	NR	6.7	6.4	14.5	16.2
			p= < 0.0001		p = 0.015	p=0.007	p = 0.37	p = 0.08

CRYSTAL = Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer; EGFR = epidermal growth factor receptor; FOLFIRI = 5-fluorouracil/leucovorin/irinotecan; KRAS = Kirsten rat sarcoma viral oncogene homolog; mCRC = metastatic colorectal cancer; NR = not reported; OPUS = Oxaliplatin and Cetuximab in Fist-line Treatment of Metastatic Colorectal Cancer; OS = overall survival; ORR = overall response rate; PFS = progression free survival; PRIME = Panitumumab Randomised Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy; WT = wild type.

mCRC have been elucidated in two pivotal studies reported in mid-2012. In the first study data from serially collected samples of patients with *KRAS* WT mCRC treated with panitumumab monotherapy were examined and *KRAS* mutations were found to be evident in 9 of 24 patients(Diaz et al., 2012). This study provided evidence for emergence of *KRAS* mutations being a central secondary resistance mechanism for EGFR-targeted therapy. The second study by Misale(Misale et al., 2012) et al. demonstrated that treatment with cetuximab led to emergence of resistance attributable to the development of *KRAS* gene amplification or mutation, and resultant *KRAS* activation in *KRAS* WT CRC cell lines. The researchers also created a *KRAS* WT CRC sub-line of cells without evidence of any *KRAS* mutant clones, despite the use of highly sensitive methods of detection. This sub-line of cells was treated with increasing concentrations of cetuximab and gradual emergence of *KRAS* mutant cells that were cetuximab resistant was observed. The findings were further verified in tumour biopsy specimens from ten patients with EGFR-targeted therapy-resistant CRC. Only two of the ten tumours were found to have mutations in *KRAS* using Sanger sequencing, but a further four tumours were found to have *KRAS* mutations with the use of more sensitive methodology including next generation sequencing (NGS). The same methods were used to examine pre-treatment tumours in the six patients who had tumours available, and all were *KRAS* WT. Furthermore, serial plasma samples collected from patients treated with cetuximab demonstrated the emergence of *KRAS* mutations up to 10 months before cetuximab resistance was detected clinically. Intriguingly, contrary to relative homogeneity of *KRAS* mutations associated with primary resistance to anti-EGFR-mABs, secondary resistance was found to be more complex; more than one mutations at the time of resistance emerged at the site of relapse.

Copy number changes such as genes encoding RTKs were also thought to be associated with secondary resistance to anti-EGFR mABs. An interesting study in cetuximab-resistant CRC cell lines identified *ERBB2* amplification a novel means of acquired resistance(Yonesaka et al., 2011). Resistant cell clones were seen to have over-expression of *ERBB2* and activation of extracellular signal-related kinase (ERK1/2) whereas reduction in *ERBB2* restored sensitivity to cetuximab. Furthermore, higher levels of heregulin, a ligand that binds *ERBB3* and *ERBB4*, were found to be associated with cetuximab resistance. The same group validated these pre-clinical observations in a retrospective clinical analysis, where amplified levels of *ERBB2* and increased levels of heregulin in CRC tumours were linked with both primary and acquired resistance to cetuximab.

MET overexpression has been found to be predictive of primary resistance to cetuximab in mCRC in a retrospective clinical study including 73 patients(Inno et al., 2011), and its role in the development of secondary resistance was demonstrated in further clinical reports (Bardelli et al., 2013). *MET* amplification was further proven to be clinically and pre-clinically a new mechanism of resistance to EGFR and BRAF dual/triple block combinations in BRAF-mutated colorectal cancer. Moreover, it was demonstrated that switching from EGFR to MET inhibition, while maintaining BRAF inhibition, resulted in clinical benefit in a patient after the occurrence of *MET*-driven acquired resistance (Pietrantonio et al., 2016). Whilst these data may need validation in a bigger cohort of patents, they highlight the importance of exploring this target further as it may have significant therapeutic implications. Our group validated these findings within a prospective phase II trial for the very first time, in patients treated with single agent cetuximab. It was interesting to note that up to 50% of the patients deemed to have RAS WT disease determined by conventional methodology had in fact RAS pathway aberrations resulting in lack of response to therapy(Khan et al., 2018). Proposed mechanisms of acquired resistance to EGFR-targeted mABs have been summarised in Table 3.

The findings from above studies indicate that the development of aberrations in *KRAS* is a cause of secondary resistance to EGFR-targeted therapy and that the acquired resistance could likely be a consequence of continuing mutagenesis, as well as expansion of existing *KRAS* mutant sub-clones under the selective pressure of EGFR-targeted therapy. It however remains unclear as to whether exposure to previous systemic therapies or other treatmemt modalities such as radiotherapy, contributed to priming of *RAS* mutant subclones or not- future studies will help undersatdning if evolutionary bottlenecks induced by previous lines of chemotherapy might have had a role in selecting RAS mutant clones. It is however intuitive to think that despite the biological complexities and challenges in understanding the true dynamics of evolutionary resistance mechanisms, the likely explanation can be attributed to polyclonal nature of resistance mechasnsims; a combination of a process of selection of pre-exisiting subclones and ongoing mutagenesis.

Table 3
Acquired resistance to Anti-EGFR therapy in mCRC.

First author	Resistance mechanism	Clinical validity	Number of patients (n)	Methods	Relevant comments and putative strategy to overcome resistance
Misale 2012, Bettegowda 2014	KRAS/NRAS mutations	Yes	10	Sanger sequencing, targeted deep sequencing	Mixed patients, chemotherapy combinations, retrospective analysis (Anti-EGFR + MEK)
Misale 2012	KRAS amplification	Yes	10	Sanger sequencing, targeted deep sequencing	Mixed patients, chemotherapy combinations, retrospective analysis (Anti-EGFR + MEK)
Yonesaka 2011	HER2 amplification	Yes	2	ELISA FISH	Targeted approach in retrospective cohort of 2 patients (Anti-EGFR+HER2)
Montagut 2012	EGFR S492R mutation	Yes	9	Deep targeted sequencing and RT-PCR	Chemotherapy combination in a retrospective cohort (panitumumab)
Bardelli 2013	MET amplification	Yes	3	NGS, qPCR	1 Patient treated with cetuximab + chemotherapy, 2 with panitumumab in a retrospective analysis (Anti-EGFR + MET)
Hobor 2014	TGF-α and amphiregulin expression	No		Pre-clinical	N/A
Sirvegna 2015	KRAS, MET	Yes	10	ddPCR, NGS, and BEAming	Mixed patients, chemotherapy combinations (Anti-EGFR + MET)
Russo 2016	MEK	Yes	1	NGS targeted panel	One patient retrospective analysis; off label treatment (Anti-EGFR + MEK)
Pietrantonio 2016	MET amplification	Yes	1 (further expanded MET testing on 17 archival samples with BRAF V600 E mutation)	NGS, ISH	One patient retrospective analysis; off label treatment Dual ALK + MET inhibitor
Khan 2018	KRAS, NRAS, MET, HER-2	Yes	45	ddPCR, deep NGS, commercially available Avenio panel	This study combined mathematical modelling with sophisticated sensitive methodology within the context of a phase II trial and validated the emerging RAS pathway aberrations within the plasma of prospectively collected samples at clinically relevant time points. High concordance was also found between plasma and tissue mutations

ALK = Anaplastic lymphoma kinase; ddPCR = digital droplet Polymerase chain reaction; ELISA (enzyme-linked immunosorbent assay); EGFR = epidermal growth factor receptor;ISH = in situ hybridisation; KRAS = Kirsten rat sarcoma viral oncogene homolog; NGS = next generation sequencing; (MEK) Mitogen-activated protein kinase kinase; mesenchymal–epithelial transition (MET); TGFα (Transforming growth factor alpha).

5. Discussion and future directions

5.1. Concordance of biomarkers in metastatic tumour tissue with primary tumour tissue in CRC

KRAS mutations are generally believed to be early events in CRC carcinogenesis and thus high concordance between primary tumour and metastatic lesions is expected (Vogelstein et al., 1988). A systematic review reporting on 21 studies established high concordance rate of *KRAS* mutational status between primary tumour and metastatic tumour (93%) (Baas et al., 2011). The average concordance of *BRAF, PIK3CA and PTEN* mutational status were 98%, 92% and 68% respectively. Whilst this review was reassuring in that it indicates that concordance between tumour primary and tumour metastatic sites was high, it also highlighted that this concordance was incomplete. Furthermore, recent studied established that 38–60% of the patients initially determined to have *KRAS* WT tumours were found to have detectable mutations by more sensitive techniques (Diaz et al., 2012; Misale et al., 2012), indicating either that there is genetic evolution in CRC tissue over the course of the disease, or that the methodology used is still suboptimal.

An alternative explanation is that there may be ongoing changes within metastatic tissue over time, which may indeed due to clonal expansion of pre-existing subclones of *KRAS* mutations after exposure to anti-EGFR therapy(Misale et al., 2014). At present our understanding about the true mechanisms of acquired resistance to anti-EGFR mABs has however significant limitations. This is probably partly due to difficulty in obtaining prospective tumour tissue for analysis from patients at a time of resistance to anti-EGFR mAB treatment and partly due to reliance of current evidence on retrospective examination of limited archival material in studies with small number of patients (Bardelli and Siena, 2010) **(**Table 3**).** An un-addressed question remains whether secondary resistance develops as a consequence of outgrowth within the resistant clones, selected by a Darwinian process, or whether it is as a result of process of mutagenesis over time, or both. Furthermore, it is not entirely clear as to how small subclones of *KRAS* mutation are able to influence the clinical outcomes in the presence of large WT allelic counterparts. In the next section we have attempted to offer a potential plausible explanation of this effect.

5.2. Biomarker discovery using a wider approach

Traditionally, prognostic and predictive biomarkers for systemic treatment have been identified using a hypothesis-driven approach where specific genes are pre-selected for molecular profiling and subsequently correlated with response and patient outcome(Tejpar et al., 2010). Whole exome or genome sequencing techniques have rapidly advanced over the past decade since they were originally used to characterise the human genome (Lander et al., 2001) and more time-consuming traditional gene sequencing methods have been overtaken by next generation sequencing methods, which are able to sequence the DNA of the whole exome or genome in up to 14 days at relatively low cost per amplicon(Ross and Cronin, 2011). For the purpose of detection of novel candidate somatic biomarker genes, the sequenced tumour genome should be compared both to matched germline DNA and with human reference genomes. The presence of candidate somatic mutations then require confirmation with an orthogonal sequencing assessment, such as with Sanger sequencing. The relevance of these genetic aberrations can then be assessed with subsequent functional studies. NGS has been applied in a study of primary CRC, where whole exome NGS was conducted in tumour and neighbouring non-tumour tissue in 6 patients with either microsatellite unstable or microsatellite stable CRC (Timmermann et al., 2010). Mutations were classified as being either somatic or germline, and further functional studies were performed to determine the importance of the identified somatic mutations on protein function. A much more comprehensive study in CRC was subsequently reported by the Cancer Genome Atlas Network, using NGS complemented by other analysis platforms(2012). This work highlighted the importance of wide biomarker discovery approach; however also demonstrated the challenges in interpretation of large amount of data, and in determining the functional and clinical significance of the numerous mutations that are uncovered. Taking this forward we used tissue obtained within PROSPECT-C, a prospective phase II clinical trial of single-agent anti-EGFR antibody (cetuximab or panitumumab) and subjected biopsies to whole-exome mutational analysis, genome wide DNA-copy number analysis and to RNA-sequencing to assess alternative mechanisms of response and resisatnce to anti-EGFR therapies (Khan et al., 2018; Woolston et al., 2019). We made several novel observations apart from validating the transit-amplifying(TA)/CMS2 transcriptomic CRC-subtypes as predictors of cetuximab benefit. The CMS2/TA-transcriptomic subtypes are known to be significantly associated with cetuximab sensitivity at baseline (Guinney et al., 2015). Based on the notion that mechanisms of acquired drug resistance are often similar to those conferring primary resistance, we investigated whether subtype identity may play a role in acquired resistance(Woolston et al., 2019). To that end, we first analysed PD-biopsies from tumors with prolonged benefit in which no genetic mechanism of acquired resistance were identified by whole-exome sequencing. We found that 5/7 cases (71%) showed a switch from the cetuximab sensitive CMS2 subtype to the CMS4 subtype and 4/7 (57%) showed a TA to SL subtype switch. In contrast, no CMS2/TA to CMS4/SL switches occurred in 6 tumors with primary PD. Switches from the cetuximab sensitive CMS2/TA-subtype to the CMS4/SL-subtype in the majority of PD-samples without identifiable genetic resistance drivers suggested that these may play a role in acquired cetuximab resistance(Woolston et al., 2019).

This study also demonstrated that biallelic *NF1*-inactivation and non-canonical *RAS/RAF*-aberrations were associated with primary progression. Novel *EGFR*-, *BRAF*- and *FGF10*-aberrations evolved at the time of acquired resistance. Interestingly, no genetic resistance drivers were identified in 57% of biopsies with acquired resistance when whole exome sequencing was performed, contrary to more sensitive techniques that were used with targeted panel approach on the same samples as demonstrated in the section below.

It is established that cetuximab is an IgG1 antibody which has been shown to induce immunogenic cell death that triggers antitumor immune responses in CRC mouse models. However, whether effective cetuximab treatment promotes immune responses in patients is unknown. We investigated this to explore potential opportunities to target cetuximab resistant CRCs with immunotherapy. We showed that cytotoxic immune infiltrates and immune-checkpoint expression increased after cetuximab responses, potentially providing opportunities to treat CRCs with molecularly heterogeneous acquires resistance to anti-EGFR therapies with immunotherapy. Overall we not only demonstrated novel mechanisms of resistance to anti-EGFR therapies but we showed that exploring immunotherapies in anti-EGFR resistant CRCs may offer any additional treatment approach in patients with modest thereupetic options(Woolston et al., 2019).

5.3. Intra-tumoural heterogeneity (ITH) and clinical complexity

Recent genetic studies across various tumour types have consistently shown that multiple subclones can evolve simultaneously within the same tumour (Gerlinger et al., 2014b; Matthew Ashenden et al., 2015). Indeed different intratumoural subclones are capable of following distinct evolutionary pathways leading to phenotypic diversification as demonstrated by several studies in CRC patients treated by anti-EGFR mABs (Gerlinger et al., 2014a; Misale et al., 2014). This branched evolution complicates precision medicine approaches due to subclonal variation in therapeutic vulnerabilities and prognostic markers. It also highlights the limitations in interpretation of data obtained by performing single biopsies as clinically most relevant subclones may have been overlooked. The identification of driver mutations in an

individual tumour is further complicated as only a limited number of common drivers have been identified for most tumour types. Moreover, as discussed previously, most known driver genes have been identified from retrospectively analysed studies of primary tumours, yet distinct drivers may be relevant for successful growth in metastatic sites or after drug therapy. In the later case, studies that identified such drivers have limitations both in terms of retrospective analysis and small numbers. Thus, current driver mutation catalogues are likely to be incomplete.

True insight into cancer evolutionary dynamic is only possible by detection of intratumoural subclones and temporal changes in clonal composition. Highly sensitive techniques such as deep NGS and digital droplet PCR (ddPCR) applied to multiple spatially separated samples collected from the same tumour lesion, or from the primary tumour and associated metastases, allow the interrogation of spatial subclonal structures and metastasis evolution. ITH and the evolution of drug resistant subclones in patients with mCRC have been examined by liquid biopsy approaches (Bardelli et al., 2013; Katsila et al., 2014; Misale et al., 2014). However, more robust prospective tissue collection studies are required in order to validate the findings of smaller studies such that clinical application and interpretation of data becomes less challenging (Misale et al., 2012; Valtorta et al., 2013).

5.4. Dense ctDNA sampling combined with mathematical modelling and its predictive value

Personalising cancer medicine requires predicting the course of the disease in individual patients. Mathematical modelling has been used to predict the evolution of resistant cancer cells(Anderson and Quaranta, 2008; Beerenwinkel et al., 2015; Gerlinger et al., 2012). Previous studies have shown that by the positive selection pressure of anti-EGFR treatment small and often undetectable RAS-mutant subpopulations at baseline expand leading eventually to treatment resistance(Diaz et al., 2012; Misale et al., 2012; Siravegna et al., 2015) In other cancers, like breast and NSCLC, prospective trials have shown liquid biopsies can identify plasma cell-free DNA (cfDNA) released from cancer cells that exhibit treatment resistant mutations before clinical progression occurs (Abbosh et al., 2017; Garcia-Murillas et al., 2015). However large variability in timescales between patients has so far limited the techniques predictive utility.

cfDNA has several inherent benefits compared to tumour biopsies. As it involves only a peripheral blood draw, cfDNA analysis can be performed repeatedly throughout treatment with little inconvenience to the patient. This allows real-time assessment of response to treatment and identify emerging resistance mechanisms. Importantly it analyses DNA fragments from all tumour cells and therefore can detect emerging resistant mechanisms in tumour subclones not amenable to biopsy(Diaz and Bardelli, 2014; Russo et al., 2016; Siravegna et al., 2017; Strickler et al., 2018). Therefore, cfDNA analysis has potentially greater sensitivity to identify mechanisms of resistance, greater patient tolerability and more clinical utility than serial tumour biopsies.

Our group recently published data from PROSPECT-C, a prospective phase II clinical trial of single-agent anti-EGFR antibody (cetuximab or panitumumab) in 47 patients with RAS wild-type [WT] mCRC(Khan et al., 2018). In this study two cohorts of patients were identified within the PROSPECT-C study. In the first cohort, twenty-two consecutive patients with *KRAS/NRAS* WT chemo refractory mCRC were analysed. All patients were treated with anti-EGFR mAb monotherapy at the Royal Marsden Hospital. Twenty patients (91.2%) received two or more lines of therapy previously. An expanded cohort of 16 patients treated with single agent cetuximab within PROSPECT-C study was also examined (Khan et al., 2018). In the first cohort, where digital droplet PCR (ddPCR) and deep NGS techniques were used, detection of *RAS* pathway aberrations in baseline ctDNA was significantly associated with inferior PFS (HR = 3.41; CI = 1.24–9.37; p = 0.02), worst OS (HR = 2.78; CI = 1.09–7.11; p = 0.03), and showed also a trend towards poor Response Rate (RR) (0% vs. 36.4%; p = 0.09) compared to WT patients(Khan et al., 2018). Interetsingly in order to corroborate these findings, data from 16 patients from the second cohort in the study were analysed by using the Roche Avenio Custom panel and all the findings from first cohort were independently validated. In the next step, we performed amplicon based ultra-deep sequencing and ddPCR validation of sequential tissue biopsies collected at clinically relevant time-points in order to dissect the architecture of *RAS* pathway aberrations and to test if the evolutionary patterns observed in ctDNA were represented in tissues(Khan et al., 2018).

We demonstrated that Variant allele frequency (VAF) of detected mutants showed high concordance between NGS and ddPCR ($r^2 = 0.94$ $p < 0.0001$). It was also demonstrated that clinical assays such as COBAS have a relatively high limit of detectability, which does not allow the detection threshold of variant alleles such that these subclonal events can't be unravelled by conventional methodology(Khan et al., 2018).

Overall, in this study, genomic data from serial cfDNA samples and matched sequential tumour biopsies were combined with imaging and mathematical modelling. The group identified two practice-changing findings 1) approximately 50% of patients with mCRC considered KRAS-WT, harboured RAS aberrations before treatment and therefore would not benefit from cetuximab and 2) early accurate quantitative predictions could be made for disease progression using the model. The majority (86%) of patients whose cfDNA was analysed at disease progression showed RAS pathway alterations and these alterations could be detected before radiographic progression or CEA increases. From this a mathematical model was derived(Khan et al., 2018), that by using serial cfDNA samples could predict with reasonable accuracy the time radiographic relapse was observed by RECIST v1.1 measurements.

This model was based on assumption that the patients cancer cells were composed of population of treatment-sensitive and treatment-resistant cells with determined growth and death rates. Initially resistant population of cells would expand undetected under selective pressure from anti-EGFR therapy until reaching the assay mutant-sensitivity threshold. Once detected over a sufficient number of time-points to infer both frequency and rate of progression the mathematical model could be applied to predict clinical disease progression. Therefore, the predictive power of the model was increased with more frequent blood draws, with a minimum of once every 4 weeks shown to be needed to have predictive utility. Similarly, the higher the mutation sensitivity of the assay the earlier the predictions could be made(Khan et al., 2018).

Applied clinically this would provide a window of opportunity for clinicians to take patient-specific treatment decisions. Not only could the exact genetic aberration(s) be identified by cfDNA analysis for a patient, by combining it with mathematical modelling the time to clinical progression can also be predicted sufficiently early to guide clinical decisions. The predictive capability of relatively simple mathematical model was remarkable but as the study was limited to assessment of the *RAS* pathway and a few other-cancer related genes other genetic and epigenetic mechanisms that play a role in the emergence of acquired resistance to anti-EGFR therapy will have been ignored. Furthermore the model awaits prospective validation in future trials.

Despite this, cfDNA can identify likely non-responders to anti-EFGR therapy and by combining serial samples with mathematical modelling predict tumour progression. Both of which promise significant clinical and economic benefits.

6. Conclusion

Sensitive methodology in combination with sophisticated bioinformatics has unravelled the subclonal composition of individual samples. Although the knowledge about the local clonal composition is growing exponentially, spatially demarcated subclones stimulated by selective pressure of therapies poses a significant therapeutic challenge. Further studies involving serial biopsies coupled with plasma samples taken at

clinically relevant turning points from individual patients treated with targeted therapies are therefore important in furthering our knowledge about genomic plasticity and evolutionary tumour dynamics. Development of minimally invasive technologies with sufficient sensitivity for ITH detection is necessary to minimise patient discomfort, biopsy risks and the difficulty in profiling spatially separated subclones by single biopsies.

Authors contributions

KK, NV, CD, SR, DW, NS, IC and DCrecruited patients in the PROSPECT-C trial, which has been cited and discussed in this review article. KK, NV, DC and CD wrote the manuscript. All the authors approved the manuscript. All authors contributed equally to this work in designing and writing this review.

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Declaration of Competing Interest

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