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Original Research

Genetic polymorphisms in angiogenesis-related genes are associated with worse progression-free survival of patients with advanced gastrointestinal stromal tumours treated with imatinib

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Abstract ***Background:*** Imatinib 400 mg per day is first-line therapy for patients with gastrointestinal stromal tumours (GISTs). Although clinical benefit is high, progression-free survival (PFS) is variable. This study explores the relationship of single nucleotide polymorphisms (SNPs) in genes related to imatinib pharmacokinetics and pharmacodynamics and PFS in imatinib-treated patients with advanced GIST.
Methods: In 227 patients a pharmacogenetic pathway analysis was performed. Genotype data from 36 SNPs in 18 genes were tested in univariate analyses to investigate their relationship with PFS. Genetic variables which showed a trend ($p < 0.1$) were tested in a multivariate model, in which each singular SNP was added to clinicopathological factors.
Results: In univariate analyses, PFS was associated with synchronous metastases ($p = 0.0008$) and the mutational status ($p = 0.004$). Associations with rs1870377 in *KDR* (additive model, $p = 0.0009$), rs1570360 in *VEGFA* (additive model, $p = 0.053$) and rs4149117 in *SLCO1B3*

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(mutant dominant model, 0.027) were also found. In the multivariate model, significant associations and trends with shorter PFS were found for synchronous metastases (HR 1.94, p = 0.002), *KIT* exon 9 mutation (HR 2.45, p = 0.002) and the SNPs rs1870377 (AA genotype, HR 2.61, p = 0.015), rs1570360 (AA genotype, HR 2.02, p = 0.037) and rs4149117 (T allele, HR 0.62, p = 0.083).

Conclusion: In addition to *KIT* exon 9 mutation and synchronous metastases, SNPs in *KDR*, *VEGFA* and *SLCO1B3* appear to be associated with PFS in patients with advanced GIST receiving 400-mg imatinib. If validated, specific SNPs may serve as predictive biomarkers to identify patients with an increased risk for progressive disease during imatinib therapy.

1. Introduction

Imatinib mesylate (Gleevec®, Glivec®) is first-line therapy for chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GISTs) [1,2]. It has revolutionised the treatment of both malignancies by achieving significant survival benefit with limited toxicity [3]. Clinical response to this oral tyrosine kinase inhibitor (TKI) is determined by somatic mutations, as well as by germline genetic variations [4,5]. Single nucleotide polymorphisms (SNPs) are the most common germline genetic variations. SNPs can have various functional effects, ranging from silent mutations to affecting gene expression and enzyme function. The pharmacokinetics and pharmacodynamics of imatinib may be changed in patients carrying SNPs in genes encoding for enzymes and target proteins involved in imatinib pharmacology.

GIST is a mesenchymal tumour of the digestive tract, often caused by gain-of-function mutations in the genes encoding for KIT or PDGFR-α [6–8]. *KIT* mutations are routinely screened in GIST to predict imatinib efficacy which is dependent on the location of the *KIT* mutation [4]. Disease progression has also been associated with clinical factors, such as the location of the primary tumour [9,10].

In CML treatment, complete cytogenetic response to imatinib has been associated with SNPs in genes encoding for enzymes which have a role in imatinib metabolism. Also, polymorphisms in the genes encoding for the efflux transporter ABCG2 (rs2231137) and for the influx transporter SLC22A1 (rs683369) have been associated with poor response and progression to advanced disease, respectively [5]. In 54 patients with advanced GIST who were treated with imatinib, associations have been reported for SNPs in *SLC22A4* (rs1050152) and *SLC22A5* (rs2631367 and rs2631372) and time to progression [11]. Since this report, no similar studies have been published. A review highlighting SNPs found in relation to imatinib in CML and GIST has been published elsewhere [12].

This study aims to investigate the relationship of genetic variants in genes encoding proteins involved in the pharmacokinetics and pharmacodynamics of imatinib and efficacy in patients with locally advanced and metastatic GIST.

2. Methods

2.1. Patients

For this exploratory retrospective study, GIST patients were included who had been treated in four Dutch referral centres. All patients had a histologically proven GIST and documented non-curative disease, being either non-resectable locally advanced or metastatic disease at the time of start of imatinib. Patients started imatinib therapy in a dose of 400 mg once daily between January 2001 and May 2013 and follow-up lasted until July 2014. All patients had to be treated until the first treatment evaluation, with the exception of patients with clinical progression before this moment. Patients with *KIT* exon 9 mutation were retained in the analysis despite having received imatinib in a 400 mg daily dose, as the objective of the study was to test the pharmacogenetic effects of 400 mg daily, and a dose of 800 mg daily will induce more toxicity. Furthermore, it is common practice in the Netherlands to start with imatinib 400 mg daily in case of a *KIT* exon 9 mutation if the tumour load is low and a patient is asymptomatic, and only escalate to 800 mg in case of progressive disease.

DNA was obtained from residual blood samples or, in the Erasmus MC Cancer Institute, after specific informed consent was obtained. Samples were stored at −20 °C until genotyping. In one location, serum of these samples was stored. If a residual blood or serum sample was not available, DNA was obtained from residual formalin-fixated paraffin-embedded (FFPE) specimen. All samples were anonymised by a third party and the Code for Proper Secondary Use of Human Tissue was adhered to (www.federa.org/codes-conduct) [13].

2.2. SNP selection

SNPs in genes related to imatinib pharmacokinetics and pharmacodynamics were selected using a pathway

approach [14]. The literature was screened for SNPs in relevant genes. Using Haploview and HapMap data (release 28), SNPs in linkage disequilibrium (>95%) were identified to select candidate SNPs. SNPs were included if the minor allele frequency was at least 0.1. In addition, the NIEHS database was used to select the SNPs with an expected functional change. A total of 36 SNPs in 18 genes were included, as shown in Table 1.

2.3. Genotyping

DNA was isolated from blood (197 patients), serum (20 patients) using the MagnaPure Compact (Roche Diagnostics, Almere, the Netherlands) or from FFPE samples (10 patients) using the Tissue Preparation System (Siemens Diagnostics, The Hague, The Netherlands) and stored at −20 °C. For optimal genotyping results, DNA isolated from serum and FFPE samples was pre-amplified using real-time PCR genotyping assays as described before [15]. A custom-made array was developed for the QuantStudio 12K Flex Real-time PCR system (Life Technologies, Bleiswijk, the Netherlands) and DNA was genotyped according to the manufacturer's protocol. To achieve a satisfactory call rate for all SNPs (>90%), a number of SNPs were subsequently genotyped using commercially available real-time PCR genotyping assays (Life Technologies, Bleiswijk, the Netherlands) according to the manufacturer's protocol or in-house developed Pyrosequencing assays (Qiagen, Venlo, the Netherlands).

Table 1
Selected SNPs in pharmacokinetics and pharmacodynamics of imatinib.

Gene	Rs number	Chromosome	Allele change	Change type
In pharmacokinetics				
ABCG2	rs2231137	4	G/A	Splicing
ABCG2	rs2231142	4	C/A	Splicing
SLC22A5	rs2631367	5	C/G	TFBS
SLC22A5	rs2631370	5	T/C	TFBS
SLC22A5	rs2631372	5	C/G	TFBS
SLC22A1	rs628031	6	G/A	Splicing
SLC22A1	rs683369	6	C/G	Splicing
SLC22A1	rs6935207	6	G/A	TFBS
ABCB1	rs1045642	7	C/T	Splicing
ABCB1	rs868755	7	G/T	Splicing
ABCB1	rs28656907	7	C/T	TFBS
SLC22A4	rs1050152	5	C/T	Splicing
CYP3A4	rs2740574	7	A/G	TFBS
POR	rs1057868	7	C/T	nsSNP
ABCC2	rs717620	10	C/T	TFBS
CYP1A1	rs1048943	15	A/G	nsSNP
CYP1A2	rs762551	15	A/C	TFBS
SLCO1B3	rs4149117	12	G/T	Splicing
In pharmacodynamics				
PDGFRA	rs1800810	4	C/G	TFBS
PDGFRA	rs1800812	4	G/T	TFBS
PDGFRA	rs1800813	4	A/G	TFBS
PDGFRA	rs2228230	4	C/T	Splicing
PDGFRA	rs35597368	4	C/T	Splicing
KDR	rs1870377	4	A/T	nsSNP
KDR	rs2071559	4	C/T	TFBS
KDR	rs2305948	4	C/T	nsSNP
VEGFA	rs1570360	6	G/A	TFBS
VEGFA	rs2010963	6	G/C	TFBS
VEGFA	rs25648	6	C/T	Splicing
VEGFA	rs3025039	6	C/T	miRNA
VEGFA	rs699947	6	A/C	TFBS
VEGFA	rs833061	6	C/T	TFBS
FLT4	rs6877011	5	C/G	miRNA
RET	rs1799939	10	G/A	Splicing
FLT3	rs1933437	13	T/C	Splicing
FLT1	rs7993418	13	A/G	Splicing

Selected SNPs in pharmacokinetics and pharmacodynamics of imatinib: Splicing, splicing modifying; TFBS, transcription factor binding site, nsSNP, non-synonymous SNP; miRNA, micro RNA alteration.

The average call rates did not differ significantly between blood, serum or FFPE samples (99.4%, 96.5% and 95.4%, respectively). All 36 SNPs had a call rate of >90%, 32 of which had >95%. Out of 36 SNPs, 31 were in the Hardy–Weinberg equilibrium (HWE) and the remaining 5 SNPs were so if just 2 patients (0.9%) had another genotype, meaning that allele frequency is not different from expected. In this patient cohort, the minor allele frequencies were in accordance to those reported in the NCBI database. To explore haplotypes in the study population, Haploview 4.2 [16] and Plink 1.7 [17] were used. SNPs in the same gene were considered to be in a haplotype in case D′ was at least 95%.

2.4. Statistics

Clinical variables were collected from patient files. Progression-free survival (PFS) was the primary end-point and defined as the time between the date of start of imatinib treatment and the date of progressive disease, according to clinical progression or to RECIST 1.1 definition of progressive disease. If patients were still on treatment at the last date of follow-up, PFS was censored at that date. The secondary end-point overall survival (OS) was defined as the time between the date of start of imatinib treatment and death due to GIST. OS was censored at the last date of follow-up if a patient was alive at that time, or a day before death if a patient had died due to an unrelated illness.

The clinical variables age, sex, synchronous metastases and mutational status (either *KIT* exon 11, *KIT* exon 9 or an 'other' group consisting of other mutations in *KIT, PDGFRA* or 'wild-type') were tested univariately with Cox regression or Kaplan–Meier analysis. These factors were included in the multivariate analysis, as they were deemed to affect imatinib efficacy. SNPs and haplotypes were univariately tested with Kaplan-Meier analysis for an association with PFS and OS. If univariate analyses showed a trend for a difference in survival ($p < 0.1$), these genetic factors were selected for the inclusion into the multivariate Cox regression model. In the multivariate model, the effect of combined clinical factors was calculated without inclusion of SNPs. To determine the impact of SNPs,

singular SNPs were added to the combined clinical factors. SNPs were tested in the additive model, unless frequency of mutant homozygote patients did not allow for this. Variables with $p < 0.05$ in the multivariate analyses were considered statistically significant. Due to the explorative nature of this study no correction for multiple testing was performed. SPSS version 20 (IBM Corp., Armonk, NY, United States) was used.

3. Results

3.1. Study population

A total of 365 patients were screened for study selection, but 68 patients had imatinib only as neo-adjuvant treatment, 41 patients had imatinib only as adjuvant treatment, in 1 patient the indication was unclear. Of the remaining 255 patients who received imatinib for locally advanced and metastatic GIST 28 had imatinib in another dose than 400 mg once daily. Therefore 227 patients were included in the study. The baseline characteristics of the study population are shown in Table 2. In 69 patients (39.2%) metastases were found at diagnosis, and in 137 patients (60.4%) either metachronous metastases or a locally advanced relapse developed in time. The median PFS for the study population was 39.0 months (95% confidence interval (CI): 27.4–50.6 months) and the median OS 86.5 months (95% CI: 70.8–102.2 months). At the time of analysis, 116 patients (51.1%) had progressive disease and 80 patients (35.2%) had died due to GIST. The median time of follow-up was 71 months, as calculated by the reversed Kaplan–Meier estimator.

Table 2
Baseline characteristics of study population.

		Number	%
Age at diagnosis	Median, in years	59.1	
Sex	Male	139	61.2
	Female	88	38.8
WHO performance score at start of imatinib	0–1	189	83.3
	2–3	8	3.5
	Unknown	30	13.2
Previous operation for GIST	Yes	158	30.4
	No	69	69.6
Mutation found	KIT exon 11	110	48.5
	KIT exon 9	22	9.7
	Other	54	23.8
	Unknown	41	18.1
Metastases or relapse with locally advanced disease	Synchronous metastases	89	39.2
	Metachronous or relapse	137	60.4
	Unknown	1	0.4

Baseline characteristics of 227 advanced GIST patients; other mutation: KIT exon 13 (3), KIT exon 14 (1), KIT exon 17 (2), PDGFR exon 12 (4), PDGFR exon 18 (4), 'wild-type' (40).

PFS was significantly longer in patients without synchronous metastases ($p = 0.0008$) and in patients who had a *KIT* exon 11 mutation as compared with *KIT* exon 9 ($p = 0.004$), while age and sex did not show an association, as shown in Table 3. Overall survival was longer in females ($p = 0.042$) and if metastases were absent at diagnosis ($p = 0.0002$), but not with other selected clinical variables, as shown in Table 4.

3.2. Pharmacogenetic factors associated with PFS

In the univariate analysis of PFS, three SNPs related to the pharmacodynamics of imatinib showed (a trend for) an association with survival. These were for rs1870377 in *KDR* (TT versus AT versus AA, $p = 0.0009$), rs1570360 in *VEGFA* (GG versus GA versus AA, $p = 0.035$) and rs4149117 in *SLCO1B3* (GG versus GT + TT, $p = 0.027$), see Table 3.

In the multivariate analysis, the combined clinical factors were associated with shorter PFS in the case of synchronous metastases and a *KIT* exon 9 mutation (HR 1.94, $p = 0.002$ and HR 2.45, $p = 0.002$, respectively). When one of the selected SNPs was added to this model, the AA genotype in rs1870377 and the AA genotype in rs1570360 were associated with shorter PFS (HR 2.61, $p = 0.037$ and HR 2.02, $p = 0.015$, respectively), whereas GT or TT genotype in rs4149117 showed a trend for longer PFS (HR 0.62, $p = 0.083$).

3.3. Pharmacogenetic factors associated with OS

In the univariate analysis of OS, a trend for association was seen in rs1870377 in *KDR* (TT versus AT versus AA, $p = 0.057$) and a statistically significant association for rs4149117 in *SLCO1B3* (GG versus GT + TT, $p = 0.030$), see Table 4. In the multivariate model only synchronous metastases was associated with OS (HR 2.71, $p = 0.0001$), whereas a *KIT* exon 9 mutation showed a trend for worse survival (HR 1.94, $p = 0.065$). Addition of a SNP to the combined clinical factors showed trends for shorter survival in case of the *AA* genotype in rs1870377 (HR 2.69, $p = 0.054$) and longer survival for the GT or TT genotype in rs4149117 (HR 0.54, $p = 0.081$).

4. Discussion

This exploratory pharmacogenetic study shows that SNPs in the genes encoding for VEGFA, KDR (also known as VEGFR2) and SLCO1B3 (also known as OATP1B3) are associated with PFS in patients with advanced GIST treated with 400-mg imatinib once daily. To the best of our knowledge, this cohort of 227 GIST patients is the largest patient group in which the pharmacogenetics of imatinib was explored. The SNP selection for this study was performed using a candidate gene approach based on imatinib pharmacology and

Table 3
Univariate and multivariate analyses of progression-free survival of advanced GIST with 400-mg imatinib.

		N patients	Univariate Kaplan–Meier analyses			Multivariate Cox regression analyses		
			Median PFS	95% CI	*p* value	HR	95% CI	*p* value
Clinical factors								
Age		(HR per year increase = 0.998)		0.984–1.013	0.812	0.9993	0.98–1.02	0.936
Sex	Male	139	35.8	24.8–46.9	0.201	1		0.348
	Female	88	47.9	2.9–92.8		0.82	0.53–1.25	
Metastasis at diagnoses	Absent	137	60.9	18.2–103.7	0.0008	1		0.002
	Present	89	24.9	18.5–33.8		1.94	1.28–2.94	
Mutation	KIT exon 9	22	19.1	5.2–32.9	0.004	2.45	1.40–4.30	0.002
	KIT exon 11	110	44.3	32.6–57.9		1		
	* other group	54	26.2	17.3–35.9		1.34	0.86–2.11	0.201
Genetic factors								
rs1870377 (*KDR*)	TT vs	132	37.7	29.8–45.6	0.0009	1		
	AT	78	50.6	28.5–72.6		0.76	0.48–1.19	0.225
	vs AA	11	8.7	3.0–14.4		2.61	1.06–6.43	0.037
rs1570360 (*VEGFA*)	GG vs	109	39.4	23.3–55.5	0.053	1		
	GA	81	60.9	1.7–120.2		0.67	0.42–1.08	0.102
	vs AA	29	28.0	14.7–41.4		2.02	1.15–3.56	0.015
rs4149117 (*SLCO1B3*)	GG vs	161	28.5	19.1–37.9	0.027	1		
	vs GT + TT	** 48	50.2	45.6–53.8		0.62	0.36–1.06	0.083

Univariate and multivariate analyses of progression-free survival of GIST with 400-mg imatinib: only univariate analyses with $p < 0.1$ shown; HR > 1.0 indicates association with worse survival and vice versa; in the multivariate Cox regression, the effect of combined clinical factors are reported without inclusion of SNPs and SNP results are presented for the singular SNP added to the model of combined clinical factors.
Abbreviations: PFS, progression-free survival; survival in months; 95% CI, 95% confidence interval; HR, hazard ratio.
*Other group consists of other mutations in KIT, PDGFR or 'wild-type'; **only 2 patients were homozygote mutant for rs4149117.

expected functionality. This, however, does not exclude the possibility that the SNPs which show an association with PFS, are in fact independent prognostic biomarkers.

So far, only one study exploring the effects of SNPs in genes related to imatinib pharmacokinetics on its efficacy was performed in patients with advanced GIST. This study investigated 31 SNPs in a population of 54 patients [11]. SNPs in *SLC22A4* (rs1050152) and *SLC22A5* (rs2631367 and rs2631372) were associated with time to progression, independent of mutational status, tumour size, age and sex. These SNPs were also tested in the present study, but univariate tests with the additive model did not show a trend for an association

Table 4
Univariate and multivariate analyses of overall survival of advanced GIST with 400-mg imatinib.

		N patients	Univariate Kaplan–Meier analyses			Multivariate Cox regression analyses		
			median OS	95% CI	*p* value	HR	95% CI	*p* value
Clinical factors								
Age	(HR per year increase = 0.9999991)			0.999–1.000	0.971	1.0005	0.98–1.02	0.960
Sex	Male	138	80.1	62.2–97.8	0.042	1		0.327
	Female	86	-#	-##		0.77	0.46–1.29	
Metastasis at diagnoses	Absent	135	119.1	-##	0.0002	1		0.0001
	Present	88	66.6	50.7–82.4		2.71	1.63–4.50	
Mutation	KIT exon 9	22	71.8	57.5–86.0	0.419	1.94	0.96–3.93	0.065
	KIT exon 11	125	89.8	73.0–106.7		1		
	* Other group	56	80.9	-##		1.11	0.65–1.91	0.702
Genetic factors								
rs1870377 (*KDR*)	TT vs	132	86.4	64.0–108.8	0.057	1		
	AT	76	100.6	67.9–133.3		0.87	0.51–1.51	0.632
	vs AA	10	67.3	24.4–110.1		2.69	0.98–7.38	0.054
rs4149117 (*SLCO1B3*)	GG vs	158	75.7	60.5–90.9	0.030	1		0.081
	GT + TT	** 48	-#	-##		0.54	0.27–1.08	

Univariate and multivariate analyses of overall of GIST with 400-mg imatinib: only univariate analyses with $p < 0.1$ shown; HR > 1.0 indicates association with worse survival and vice versa; in the multivariate Cox regression the effect of combined clinical factors are reported without inclusion of SNPs and SNP results are presented for the singular SNP added to the model of combined clinical factors.
Abbreviations: OS, overall survival; survival in months; 95% CI, 95% confidence interval; HR, hazard ratio. #, median not reached; ##, 95% CI not computed.
*Other group consists of other mutations in KIT, PDGFR or 'wild-type'; **only 2 patients were homozygote mutant for rs4149117.

with survival. Possibly, the small sample size can account for this discrepancy.

Several SNPs in vascular endothelial growth factor A (*VEGFA*) were included in this study. *VEGFA* plays a crucial role in inducing angiogenesis. Compared with weak or non-expressers, high VEGF expression in GIST has been associated to inferior PFS during imatinib therapy [18]. Also, imatinib may lead to decreased VEGF expression in a subset of patients [18]. In this study, rs1570360 in *VEGFA* was associated with PFS. Other SNPs in *VEGFA* such as rs699947 have been associated with a reduced effect of imatinib in CML patients, but none other of the tested SNPs showed a significant association in this study population [19]. In this study, rs7993418 in *FLT1* (encoding for vascular endothelial growth factor receptor 1) and rs6877011 in FLT4 (encoding for the receptor of vascular endothelial growth factor C and D) did not show an association with survival [20].

The rs1870377 SNP in kinase insert domain receptor (KDR, also known as VEGFR2) was associated with shorter PFS (and less so with OS) in the present study population. This may be due to increased micro-vessel density seen in tumours with this SNP mutation [21]. The effect of enhanced tumour angiogenesis may be stronger in terms of increased nutrient supply as compared with improved accessibility for imatinib. Having a variant in this SNP has also been shown to increased GIST susceptibility, pointing to a role of VEGF in GIST biology [22]. A study investigating SNPs in *KDR* for an effect on GIST relapse rate did not show a similar effect, in contrast to a study with CML patients, which reported better clinical outcome for patients with the wild-type genotype in rs1870377 [19,20].

Patients with at least one T allele in rs4149117 in *SLCO1B3* had a trend for longer OS. The solute carrier organic anion transporter family member (SLCO) 1B3 is an influx transporter with imatinib as a substrate [23]. A study performed in CML patients reported that the frequency of patients with the *TT* genotype was higher in the responder group than in the non-responder group [24]. These results are in line with a study from Japan, which found enhanced transporter function in patients with the *TT* genotype, as measured by higher intracellular imatinib levels [25].

As previously reported, the effect of the oncogenic somatic mutation on imatinib efficacy was also found in this study. Tumours with a *KIT* exon 11 mutation were more sensitive to imatinib compared with tumours with a *KIT* exon 9 mutation [4]. Patients with a *KIT* exon 9 mutation received imatinib at a dosage currently considered too low, but this was corrected for in the multivariate analysis. Presence of synchronous metastases was clearly associated with reduced survival. These metastases may be considered heterogeneous and some clones will progress despite imatinib activity in the majority of GIST lesions [26]. Other clinical factors were not associated with survival, even though factors such as the primary tumour site have been reported in other studies [9].

Remarkably, SNPs in the pharmacokinetic genes encoding for ABCB1, ABCG2, SLC22A1, SL22A5 or CYP3A4 were not associated with a difference in survival, despite previous, sometimes conflicting, reports [5,10,11,19,20,27–29]. A hypothetical explanation may be that most patients had an imatinib serum level higher than the threshold needed for clinical activity, negating any effects that these SNPs may have on the actual serum level above this threshold.

This study has limitations, mainly due to the retrospective nature of the data. In addition, DNA derived from blood was not available for all patients. FFPE samples were used instead, as it has been demonstrated to be a valid proxy for DNA from peripheral blood [30]. Out of the 36 SNPs tested, 5 were not in HWE. These SNPs were retained in the analyses, as an allele change in only 2 patients would mean these SNPs are in HWE, and patient selection due to the retrospective nature of the study was considered the most plausible reason.

This study investigated the associations of polymorphisms in genes related to the pharmacokinetics and pharmacodynamics of imatinib in the treatment of advanced GIST. One SNP in the pharmacokinetic pathway (rs4149117 in *SLCO1B3*) and two SNPs related to pharmacodynamics (rs1870377 in *KDR*, and rs1570360 in *VEGFA*) were significantly associated with PFS. When replicated, these polymorphisms, together with tumour mutation and metastases, may identify patients who are most at risk of developing progressive disease and it may select patients whom may benefit from more frequent treatment evaluation or alternative first-line treatments that are currently being developed (e.g. NCT02365441).

Conflict of interest statement

None declared.

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