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Pharmacogenetic determinants of outcomes on triplet hepatic artery infusion and intravenous cetuximab for liver metastases from colorectal cancer (European trial OPTILIV, NCT00852228)

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Background: The hepatic artery infusion (HAI) of irinotecan, oxaliplatin and 5-fluorouracil with intravenous cetuximab achieved outstanding efficacy in previously treated patients with initially unresectable liver metastases from colorectal cancer. This planned study aimed at the identification of pharmacogenetic predictors of outcomes.

Methods: Circulating mononuclear cells were analysed for 207 single-nucleotide polymorphisms (SNPs) from 34 pharmacology genes. Single-nucleotide polymorphisms passing stringent Hardy–Weinberg equilibrium test were tested for their association with outcomes in 52 patients (male/female, 36/16; WHO PS, 0–1).

Results: *VKORC1* SNPs (rs9923231 and rs9934438) were associated with early and objective responses, and survival. For rs9923231, T/T achieved more early responses than C/T (50% *vs* 5%, $P=0.029$) and greatest 4-year survival (46% *vs* 0%, $P=0.006$). *N*-acetyltransferase-2 (rs1041983 and rs1801280) were associated with up to seven-fold more macroscopically complete hepatectomies. Progression-free survival was largest in *ABCB1* rs1045642 T/T ($P=0.026$) and rs2032582 T/T ($P=0.035$). Associations were found between toxicities and gene variants ($P<0.05$), including neutropenia with *ABCB1* (rs1045642) and *SLC0B3* (rs4149117 and rs7311358); and diarrhoea with *CYP2C9* (rs1057910), *CYP2C19* (rs3758581), *UGT1A6* (rs4124874) and *SLC22A1* (rs72552763).

Conclusions: *VKORC1*, *NAT2* and *ABCB1* variants predicted for HAI efficacy. Pharmacogenetics could guide the personalisation of liver-targeted medico-surgical therapies.

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The determination of non-invasive biomarkers has long been advocated for improving efficacy or reducing toxicity through helping personalise both drug selection and dose determination in cancer chemotherapy protocols (Hertz and Rae, 2015). Thus, allele frequencies of genes responsible for anticancer drug absorption, distribution, metabolism and elimination (ADME) have been related to pharmacokinetics, toxicities or response (Hertz and Rae, 2015). Classical examples of single-nucleotide polymorphisms (SNPs) associated with individual toxicities of an anticancer drug include genes UDP-glucuronosyltransferase 1A (*UGT1A*) for irinotecan, and thymidylate synthase and *DPYD* for fluoropyrimidines (Falvella *et al*, 2015; Hertz and Rae, 2015; Milano, 2016). However, cancer chemotherapy usually combines several anticancer drugs. As an example, hepatic artery infusion (HAI) of irinotecan, 5-fluorouracil (F) and oxaliplatin (O) (IFO) was recently combined with intravenous cetuximab in previously treated patients with liver metastases from colorectal cancer (LM-CRC) within European Phase II clinical trial OPTILIV (Levi *et al*, 2016). The conversion rate of previously unresectable LM to curative intent hepatectomy (R0–R1) reached 30%, and overall median survival was 25 months despite protocol application as median third-line chemotherapy (Bouchahda *et al*, 2016; Levi *et al*, 2016). Achieving early rather than late tumour shrinkage has indeed become an important goal of chemotherapy of LM-CRC, as it can translate into previously unforeseen surgical resections with prolonged survival or cure (Bismuth *et al*, 1996; Giacchetti *et al*, 1999; Adam *et al*, 2004). Hence, we used a multiple molecular typing method, using the iPLEX Agena Bioscience MassARRAY platform (Williams *et al*, 2008), in order to identify a possible genetic basis for the efficacy and toxicities of triplet HAI and i.v. cetuximab. No pharmacogenetic study has yet explored such issue for HAI drugs despite its original mechanisms of action involving the direct metastases exposure to anticancer drugs (Kemeny *et al*, 2006; Bouchahda *et al*, 2011; D'Angelica *et al*, 2015; Maeda *et al*, 2016).

PATIENTS AND METHODS

The pharmacogenetic assessment in OPTILIV was approved by the ethical committee and the national regulatory authorities in four countries. It aimed at the identification of those constitutive SNPs that would predict for success in main outcomes on OPTILIV protocol treatment.

Patients. Participants in OPTILIV protocol had a histological proof of colorectal cancer, unresectable liver metastases, wild-type *KRAS* tumour, WHO performance status of 0–1 and adequate biology (Levi *et al*, 2016). They had received one, two or three prior chemotherapy protocols. They signed informed consent for the pharmacogenetic assessment (Levi *et al*, 2016).

Treatment. All patients received OPTILIV protocol treatment consisting in biweekly administration of hepatic artery infusion of IFO, combined to intravenous infusion of cetuximab. Hepatic artery infusion was administered either as a conventional modality or according to chronomodulated delivery, according to institution experience (Bouchahda *et al*, 2016; Levi *et al*, 2016).

Treatment evaluation. Blood cell counts, serum chemistries and adverse events were monitored before each treatment course and graded according to NCI-CTCAE vs3.0 criteria. Tumour response imaging was obtained every three courses and classified according to RECIST. Patients were assessed for secondary liver surgery at iterative oncosurgical evaluations after three, six or nine cycles (Bouchahda *et al*, 2016).

Pharmacogenetics. A volume of 10 ml of whole blood was drawn in an EDTA-tube before treatment onset (between 0800 and 1000 hours) and stored at −20 °C. Sampling was occasionally performed at other times for technical reason. Genomic DNA was extracted from blood using a QIAamp DNA mini kit (Qiagen, Courtaboeuf, France). The concentration and purity of the DNA were determined by absorbance at 260 and 280 nm using a Nanovue spectrophotometer (Biochrom, Harvard Bioscience Inc, Holliston, MA, USA). The genotyping to investigate biomarkers associated with drug ADME was performed on the Sequenom Massarray platform (Sequenom, San Diego, CA, USA). The iPLEX ADME PGx panel (Sequenom) screening for 207 polymorphisms in 34 genes and 200 assays developed for screening of known, high-value target genes associated with drug metabolism and toxicity was used with Typer Assay designer software (Agena Bioscience GmbH, Hamburg, Germany) and iPLEX Gold biochemistry (Agena Bioscience GmbH). The PCR primers and the extension primers were mixed in eight unique pools, and used for amplification of target regions and interrogation of the specific base composition at the target site using single base extension.

Multiplexed PCR was performed in 5 μl volumes after DNA dilution. The amplification protocol comprised an initial incubation at 94 °C for 4 min; 45 cycles of denaturation at 95 °C for 20 s, annealing at 62 °C for 30 s and extension at 72 °C for 1 min; and final incubation at 72 °C for 3 min. Unincorporated deoxynucleoside triphosphates were dephosphorylated by the addition of 2 μl of premix including 0.3 U of shrimp alkaline phosphatase (Sequenom). The reaction mixture was incubated at 37 °C for 40 min, after which the phosphatase was inactivated by incubation for 5 min at 85 °C. Final primer extension was carried out using primer extension probes, the appropriate dNTP/ddNTP combination and 0.5 units of Thermosequenase DNA polymerase (Sigma-Aldrich Chimie Sarl, Lyon, France). Reactions were cycled at 94 °C for 2 min, followed by 40 cycles of 94 °C for 5 s and 5 cycles of 52 °C for 5 s and 80 °C for 5 s, and final incubation at 72 °C for 3 min. After addition of a cation exchange resin to remove residual salt form the reactions, 7 nl of the purified primer extension reaction was loaded onto a matrix pad of a spectroCHIP (Sequenom). SpectroCHIPs were analysed using MALDI-TOF mass spectrometer.

Pharmacokinetics. Circulating drug and main metabolite levels were determined following iterative blood sampling during the first treatment course of 11 patients on chronomodulated HAI. The serum or plasma concentrations of cetuximab, irinotecan, SN38, total and ultrafiltrated oxaliplatin, and 5-fluorouracil were determined according to Levi *et al* (2017).

Statistical considerations. The association of gene polymorphisms with toxicity and efficacy was first assessed using adequate non-parametric tests (Mann–Whitney *U*-test, Fisher exact test or Kruskal–Wallis). A *P*-value of <0.05 was considered as statistically significant. Statistical analyses were performed using SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL, USA).

Genotypic data analysis. Genomic coordinates of polymorphisms were annotated on GRCh37.p13 version of the human genome. Genotypic data obtained with the Sequenom technology have been formatted to form a compatible matrix with the software SNPAnalyzer (Yoo *et al*, 2008). Preprocessing of data was performed by removing samples with $>50\%$ missing genotype; removing also SNPs with missing genotypes over 10% and SNPs with minor allele frequency $<5\%$. This preprocessing analysis also included a Hardy–Weinberg equilibrium (HWE) test: SNPs with HWE *P*-value <0.05 were withdrawn from further analysis. Genetic association was validated using Fisher exact test with Bonferroni's multitesting corrections for controlling for false discovery rate. Analysis of linkage disequilibrium

Table 1. Main characteristics of all 52 patients and according to early response and complete macroscopic liver resection

		Early response ($n=49$)[a]			Liver resection ($n=52$)		
Patient characteristics	**All ($N=52$)**	**Yes ($n=11$)**	**No ($n=38$)**	***P***	**R0–R1 ($n=14$)**	**No resection ($n=38$)**	***P***
Age (years)							
Median (range)	59 (33–76)	57 (33–76)	60 (40–73)	0.310	49 (33–76)	60 (48–75)	0.003
Sex							
Male	36 (69.2%)	6 (18.2%)	27 (81.8%)	0.466	29 (80.6%)	7 (19.4%)	0.094
Female	16 (30.8%)	5 (31.3%)	11 (68.8%)		9 (56.3%)	7 (43.8%)	
Site of primary tumour							
Colon	40 (76.9%)	8 (21.1%)	30 (78.9%)	0.692	11 (27.5%)	29 (72.5%)	1
Rectum	12 (23.1%)	3 (27.3%)	8 (72.7%)		3 (25.0%)	9 (75.0%)	
No. of chemotherapy lines							
1	21 (40.4%)	5 (27.8%)	13 (72.2%)	0.503	10 (47.6%)	11 (52.4%)	0.006
2–3	31 (59.6%)	6 (19.4%)	25 (80.6%)		4 (12.9%)	27 (87.1%)	
WHO performance status							
WHO PS 0	31 (59.6%)	7 (24.1%)	22 (75.9%)	1	8 (25.8%)	23 (74.2%)	0.825
WHO PS 1/2	21 (40.4%)	4 (20.0%)	16 (80.0%)		6 (28.6%)	15 (71.4%)	
Synchronous metastases							
Yes	45 (86.5%)	9 (20.9%)	34 (79.1%)	0.605	11 (24.4%)	34 (75.6%)	0.370
No	7 (13.5%)	2 (33.3%)	4 (66.7%)		3 (42.9%)	4 (57.1%)	
Metastases location in liver							
Unilateral	9 (17.3%)	2 (25.0%)	6 (75.0%)	1	4 (44.4%)	5 (55.6%)	0.229
Bilateral	43 (82.7%)	9 (22.0%)	32 (78.0%)		10 (23.3%)	33 (76.7%)	
Liver involvement							
⩽25%	21 (40.4%)	5 (25.0%)	15 (75.0%)	0.740	9 (42.9%)	12 (57.1%)	0.033
>25%	31 (59,6%)	6 (20.7%)	23 (79.3%)		5 (16.1%)	26 (83.9%)	
No. of liver metastases							
Median (range)	9 (1–69)	15 (2–50)	9 (1–69)	0.169	8 (2–50)	10 (1–69)	0.538
Largest meta diameter (mm)							
Median (range)	56.5 (15–172)	50 (15–93)	59 (18–172)	0.151	37 (15–131)	60 (18–172)	0.076
No. of liver segments involved							
Median (range)	6 (1–8)	6 (1–8)	6 (2–8)	0.400	6 (1–8)	6 (1–8)	0.159
Sites involved							
Liver only	30 (57.7%)	4 (14.8%)	23 (85.2%)	0.185	8 (26.7%)	22 (73.3%)	0.961
Liver + other sites[b]	22 (42.3%)	7 (31.8%)	15 (68.2%)		6 (27.3%)	16 (72.7%)	

Abbreviations: PS = performance status; WHO = World Health Organisation.
[a]Three patients were not assessed for response.
[b]Colon, rectum, lung or lymph node.

(LD) was calculated by the LD index |D′| and retained significant polymorphisms for Pearson's correlation coefficients >0.80. The LD analysis was performed using the method of Gabriel without any limit in genetic distance parameter (Gabriel *et al*, 2002).

Pharmacokinetics analyses. For each drug and for both metabolites, maximum plasma concentration (Cmax) values were determined, with their time to reach Cmax (*t*max) values. Area under the concentration curves were calculated for each drug (Levi *et al*, 2017). The relations between these parameters and drug metabolism polymorphisms were statistically validated using analysis of variance.

RESULTS

Patient characteristics and outcomes. Fifty-two out of 64 patients (85%) with unresectable LM-CRC enrolled into the OPTILIV trial consented for and had valid sample for the study (Table 1; Figure 1). All the patients had received one to three chemotherapy protocols before OPTILIV. The main dose-limiting grade 3–4 toxicities on OPTILIV were neutropenia (40.4% of the patients), fatigue (21.2%) and diarrhea (17.3%; Table 2). Twenty patients had an objective

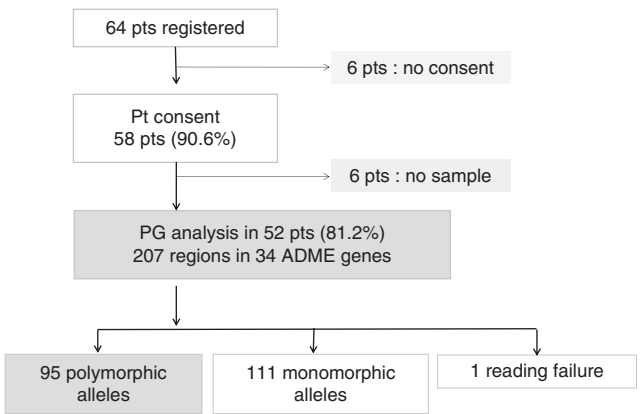

Figure 1. Consort diagram. This pharmacogenetic study involved 52 patients out of the 64 who had been registered in OPTILIV for receiving a combination of intravenous cetuximab and HAI of irinotecan, oxaliplatin and 5-fluorouracil (85% of the trial population). Thirty-four drug metabolism genes were analysed for a total of 207 candidate SNPs. Ninety-five of them were polymorphic (49.7%). Their association with clinical outcomes was investigated further.

Table 2. Characteristics of patients according main grade 3–4 toxicities over the first six courses

		Neutropenia (grade 3–4)			Diarrhoea (grade 3–4)			Fatigue (grade 3–4)			Thrombosis (grade 3–4)		
Patient characteristics	**All, *N* = 51 (100%)**	**Yes, *n* = 20 (39.2%)**	**No, *n* = 31 (60.8%)**	***P***	**Yes, *n* = 9 (17.6%)**	**No, *n* = 42 (82.4%)**	***P***	**Yes, *n* = 10 (19.6%)**	**No, *n* = 41 (80.4%)**	***P***	**Yes, *n* = 2 (3.9%)**	**No, *n* = 49 (96.1%)**	***P***
Age (years)													
Median (range)	59 (33–76)	60 (33–72)	49 (40–76)	0.557	63 (54–73)	58 (33–73)	0.053	60 (40–72)	59 (33–76)	0.963	47 (33–60)	59 (33–76)	0.083
Sex													
Male	35 (68.6%)	11 (31.4%)	24 (68.6%)	0.092	5 (14.3%)	30 (85.7%)	0.436	4 (11.4%)	31 (88.6%)	0.054	1 (2.9%)	34 (97.1%)	0.533
Female	16 (31.4%)	9 (56.3%)	7 (43.8%)		4 (25.0%)	12 (75.0%)		6 (37.5%)	10 (62.5%)		1 (6.3%)	15 (93.8%)	
Site of primary tumour													
Colon	40 (78.4%)	18 (45.0%)	22 (55.0%)	0.166	6 (15.0%)	34 (85.0%)	0.385	9 (22.5%)	31 (77.5%)	0.428	2 (5.0%)	38 (95.0%)	1
Rectum	11 (21.6%)	2 (18.2%)	9 (81.8%)		3 (27.3%)	8 (72.7%)		1 (9.1%)	10 (90.9%)		0	11 (100%)	
No. of chemotherapy lines													
1	20 (39.2%)	8 (40%)	12 (60.0%)	0.927	2 (10.0%)	18 (90.0%)	0.454	3 (15.%)	17 (85.0%)	1	2 (10.0%)	18 (90.0%)	0.149
2–3	31 (60.8%)	12 (38.7%)	19 (61.3%)		7 (22.6%)	24 (77.4%)		7 (22.58%)	24 (77.4%)		0	31 (100%)	
WHO performance status													
WHO PS 0	30 (58.8%)	12 (40.0%)	18 (60.0%)	0.891	6 (20.0%)	24 (80.0%)	0.720	6 (20.0%)	24 (80.0%)	1	1 (3.3%)	29 (96.7%)	1
WHO PS 1/2	21 (41.2%)	8 (38.1%)	13 (61.9%)		3 (14.3%)	18 (85.7%)		4 (19.0%)	17 (81.0%)		1 (4.8%)	20 (95.2%)	
Synchronous metastases													
Yes	45 (88.2%)	16 (35.6%)	29 (64.4%)	0.195	7 (16.6%)	38 (84.4%)	0.284	9 (20.0%)	36 (80.0%)	1	2 (4.4%)	43 (95.6%)	1
No	6 (11.8%)	4 (66.7%)	2 (33.3%)		2 (33.3%)	4 (66.7%)		1 (16.7%)	5 (83.3%)		0	6 (100%)	
Metastases location in liver													
Unilateral	9 (17.6%)	2 (22.2%)	7 (77.8%)	0.454	2 (22.2%)	7 (77.8%)	0.651	3 (33.3%)	6 (66.7%)	0.353	0	9 (100%)	1
Bilateral	42 (82.4%)	18 (42.9%)	24 (57.1%)		7 (16.7%)	35 (83.3%)		7 (16.7%)	35 (83.3%)		2 (4.8%)	40 (95.2%)	
Liver involvement													
⩽25%	20 (39.2%)	9 (45.0%)	11 (55.0%)	0.497	3 (15.0%)	17 (85.0%)	1	7 (35.0%)	13 (65.0%)	0.036	1 (5.0%)	19 (95.0%)	1
>25%	31 (60.8%)	11 (35.5%)	20 (64.5%)		6 (19.4%)	25 (80.6%)		3 (9.7%)	28 (90.3%)		1 (3.2%)	30 (96.8%)	
No. of liver metastases													
Median (range)	9 (1–69)	10 (3–50)	9 (1–69)	0.925	8 (1–50)	10 (1–69)	0.627	5 (1–12)	10 (1–69)	0.038	18 (5–30)	9 (1–69)	0.772
Largest meta diameter (mm)													
Median (range)	57 (15–172)	57 (18–110)	57 (15–172)	0.667	65 (25–172)	51 (15–130)	0.010	60 (18–110)	56 (15–172)	0.961	70 (39–101)	57 (15–172)	0.694
No. of liver segments involved													
Median (range)	6 (1–8)	6 (2–8)	6 (1–8)	0.670	7 (2–8)	6 (1–8)	0.901	5 (2–7)	7 (1–8)	0.031	6 (5–7)	6 (1–8)	0.860
Sites involved													
Liver only	30 (58.8%)	14 (48.3%)	15 (51.7%)	0.128	6 (20.7%)	23 (79.3%)	0.714	6 (20.7%)	23 (79.3%)	1	2 (6.9%)	27 (93.1%)	0.500
Liver + other sites[a]	21 (41.2%)	6 (27.3%)	16 (72.7%)		3 (13.6%)	19 (86.4%)		4 (18.2%)	18 (81.8%)		0	22 (100%)	

Abbreviations: PS = performance status; WHO = World Health Organisation.
[a]Colon, rectum, lung or lymph node.

responses (38.4%), which occurred after up to three courses for eleven of them. Fourteen patients (26.9%) underwent macroscopically complete LM resections (R0–R1). Median progression-free survival (PFS) was 8.6 months (6.6–10.7) and median overall survival was 21.9 months (15.0–28.7), with ten 4-year survivors (19%). Ninety-five polymorphisms were identified within the 207 reference SNP (rs) identification numbers tested (Supplementary Table S1), with 16 loci (7.7%) in 10 out of 34 ADME genes (29.4%) successfully passing the stringent filtering process, thus undergoing evaluation regarding relations to outcomes.

Association of *VKORC1* polymorphisms with early response, objective response and overall survival. Two loci (rs9923231 and rs9934438) in *VKORC1* were robustly associated with both early response and overall objective response, while SNPs in rs9923321 were also associated with overall survival (Figure 2). For rs9923231, T/T ($N = 8$) as compared to C/T ($N = 21$) had greatest chance of achieving both early response (50% *vs* 5%, $P = 0.029$; Supplementary Table S2) and 5-year survival (46% *vs* 0%, $P = 0.006$; Figure 2). Single-nucleotide polymorphisms in rs9923231 further displayed a non-statistically significant association with arterial thrombosis, while SNPs in rs7294 locus were associated with such adverse event. For rs9923231, arterial thrombosis was encountered in 77% of the T/T patients as compared to 30% of the C/C ones ($P = 0.04$). As a result, the odds ratios clearly revealed that the T/T genotypes of rs9923231 had more early responses, and more catheter thrombosis as well, and displayed a far better survival as compared to the C/T genotyped patients (Figure 2). Single-nucleotide polymorphisms in *CYP2C19* (rs12248560) and *SLC15A2* (rs1243672) were also associated with early, but not objective response or survival (Supplementary Table S2).

***N*-acetyltransferase 2 polymorphisms and conversion to resection.** Two SNPs within the *NAT2* gene (rs1801280 and rs179929) were associated to LM R0 + R1 resection, and further confirmed so, using LD analysis (Figure 3). For rs1041983, the rate of LM resections was as low as 13% and 20% for the patients with C/C and T/T, respectively, as compared to 50% for C/T (P from exact Fischer = 0.024). For rs1801280, the LM resection rate ranged from 6.25% for the C/C patients to 42.8% for the T/T ones ($P = 0.055$). Univariate analyses indicated that an increased likelihood of achieving R0–R1 resection was significantly associated with both (a) clinical factors, including a male sex, an age ⩽60 years, a liver involvement ⩽25%, a number of metastases ⩽10, a largest metastasis diameter ⩽53 mm and a single prior systemic chemotherapy protocol, and (b) *NAT2* rs1801280 T/T. Multivariate logistic regression identified *NAT2* (rs1801280) as the single independent prognostic factor of macroscopically complete LM

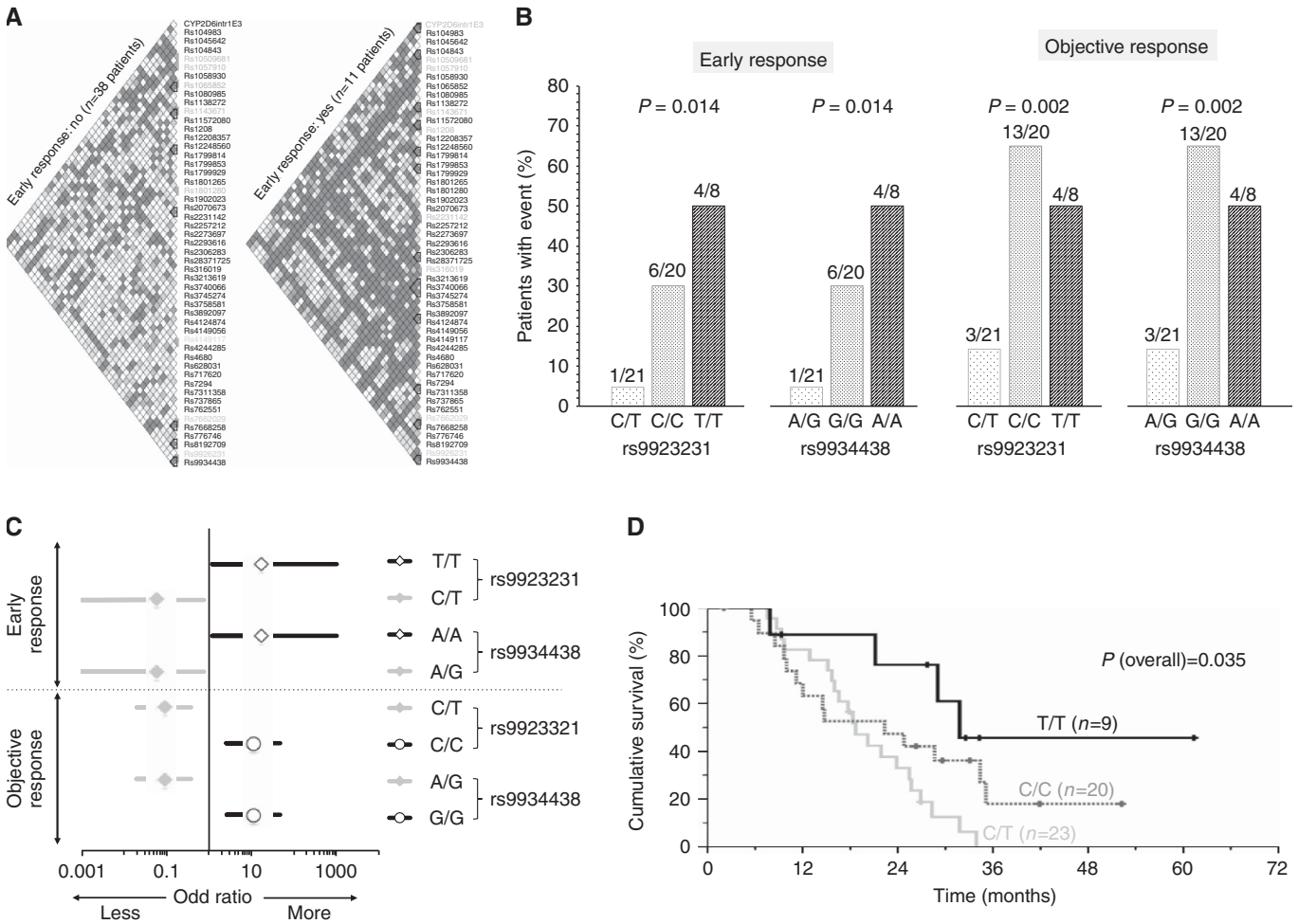

Figure 2. Associations between *VKORC1* SNPs (rs9923231 and rs9934438) and efficacy or tolerability end points of i.v. Cet and triplet HAI protocol. (**A**) Results from LD analysis on whole study population stratified according to early tumour response. After filtration with HWE test, pairwise analysis was conducted to identify blocks of LD using Gabriel method. Blocks grouping SNPs are represented by geometric triangles in black. Single-nucleotide polymorphisms flagged in blue have a correlation coefficient >0.8 in the pairwise analysis. The more intense the red colour in each heatmap cell, the higher the correlation coefficient between two SNPs in the same group of patients. Note: this analysis revealed that SNPs' correlation with no distance limit was highest in the early response group (panel on the right) as compared to the non-early response group (panel on the left). The results suggest both least genetic heterogeneity in the early responders and adequate selection of SNPs for such analysis. (**B**) Column graphs describing the relations of *VKORC1* SNPs with early and objective responses. Number of patients with response out of number of patients with corresponding genotype is indicated above each column. *P*-values are from Fischer exact. (**C**) Corresponding odds ratios. (**D**) Overall survival curves according to rs9923231 SNPs. *P*-value from log-rank test shown for overall comparison. Statistically significant differences in survival curves further documented between T/T (median, 31.8 months) and C/T (median, 18.7 months (15.0–22.3); intermediate median survival for C/C, 22.3 months (7.8–36.9). Note best efficacy for three end points in rs9923231 T/T. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

resection. The hazard ratio (T/T *vs* C/C) was 18.8 (95% CL, 1.8–209.4; $P = 0.017$).

***ABCB1* polymorphisms and PFS.** Single-nucleotide polymorphisms at both rs1045642 and rs2032582 in *ABCB1* were associated with statistically significant differences in PFS ($P = 0.026$ and 0.035, respectively; Figure 4). For rs2032582, median PFS ranged from 7.0 months for G/T ($N = 23$), as compared to 10.9 months for T/T ($N = 7$; $P = 0.036$). Moreover, disease progression occurred in all G/T or G/G patients within the 30 months following OPTILIV inclusion, while no progression was encountered for 29% of the T/T genotype. Multivariate analysis further revealed that *ABCB1* (rs2032582) genotype was an independent prognostic factor of PFS, jointly with sex, initial liver involvement and R0–R1 resection (Supplementary Table S3). Interestingly, the other *ABCB1* polymorphic SNP (rs1045642) was also associated with severe neutropenia. Thus, 75% of the T/T patients experienced grade 3–4 neutropenia, as compared to 41.9% of the C/T genotype and 9.1% of the C/C one (Supplementary Table S2; Figure 5).

Single-nucleotide polymorphisms associated with main systemic toxicities. Statistically significant associations ($P < 0.05$) of SNPs with main toxicity outcomes were found for oxydo-reduction (*CYP2E1* and HA thrombosis, *CYP2C9* and diarrhea, and *CYP2C19* and both diarrhea and fatigue), conjugation (*UGT1A6* and diarrhea, and *NAT2* and fatigue), and transport (*ABCB1* or *SLC0B3* and neutropenia, and *SLC22A1* and diarrhea; Supplementary Table S2; Figure 5). Interestingly, *ABCB1* (rs1045642) was the single-gene polymorphism that was statistically associated with both severe toxicity (grade 3–4 neutropenia) and the plasma pharmacokinetics parameters we determined during the first protocol course (Cmax of oxaliplatin and cetuximab, and estimated AUC of cetuximab) in a subset of 11 of these 52 patients (Supplementary Table S4).

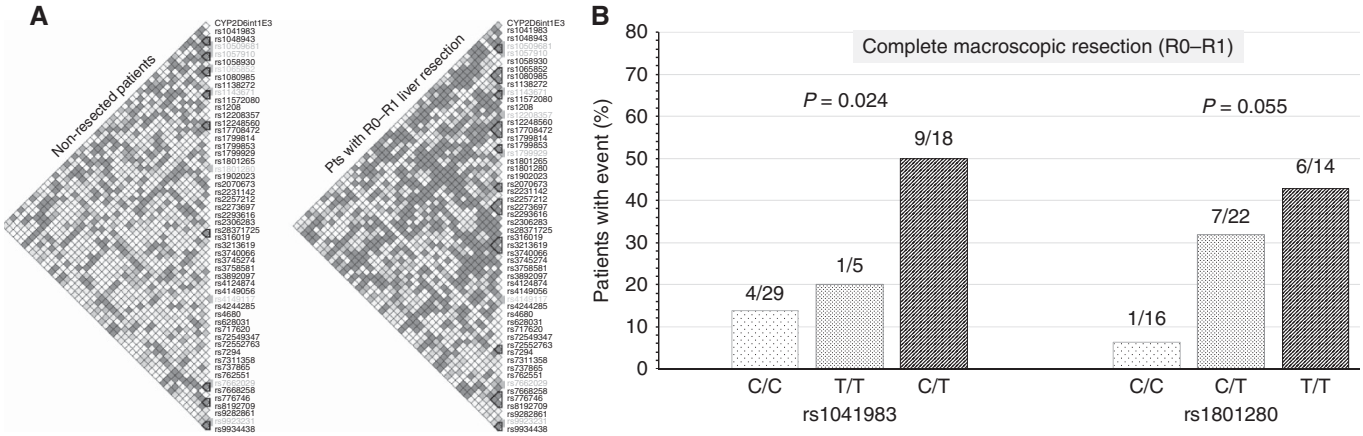

Figure 3. Associations between *N*-acetyltransferase 2 (*NAT2*) SNPs (rs1041983 and rs1801280) and macroscopically complete liver metastases resections (R0 + R1) following i.v. Cet and triplet HAI protocol. (**A**) Results from LD analysis on whole study population stratified according to R0 + R1, after application of Hardy–Weinberg method (see legend of Figure 2A). This display revealed both least genetic heterogeneity in the R0 + R1 resection group (right panel), as compared to the non-resected patient group (left panel), and adequate selection of SNPs for such analysis. (**B**) Column graphs. Number of patients with R0–R1 resections out of number of patients with corresponding genotype is indicated above each column. *P*-values are from Fischer exact. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

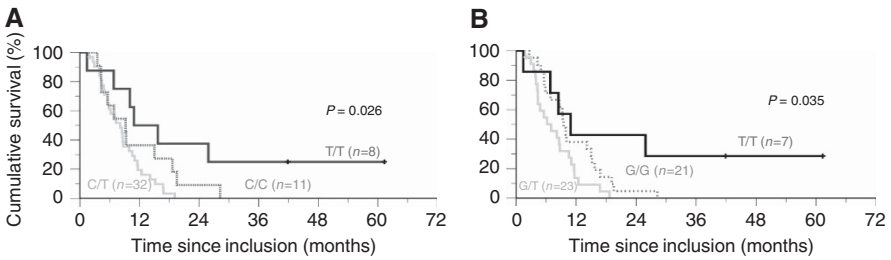

Figure 4. Progression-free survival curves according to *ABCB1* genotype at (A) rs1045642 (left) and (B) rs2032582. Number of patients per genotype in parentheses. *P*-value from log-rank test for each overall comparison. Statistically significant differences in survival curves were further documented for rs1045642 between C/T (median, 8.1 months (95% CL, 5.5–10.8)) and T/T (median, 10.9 months (3.1–18.7) ($P = 0.015$)), and for rs2032582 between G/T (median, 7.0 months (3.2–10.8)) and both G/G (9.5 months (8.5–10.5) ($P = 0.040$)) and T/T (10.9 months (4.7–17.1)).

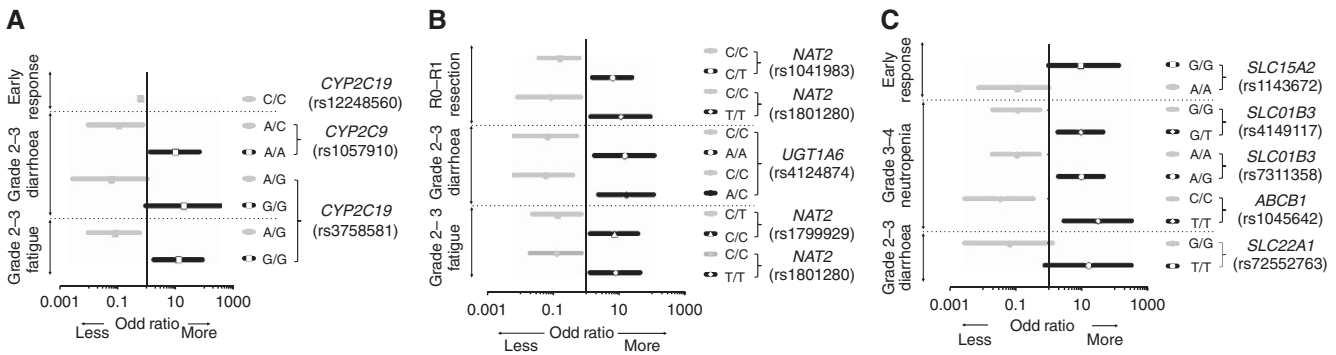

Figure 5. Relations between SNPs in selected drug metabolism genes and main OPTILIV treatment end points. Odds ratios and 95% confidence limits for statistically significant associations with early response (upper rows) and main toxicities (lower rows). (**A**) Polymorphisms in phase 1 metabolism genes (*CYP2C19* and *CYP2C9*) were associated with early response, diarrhoea and fatigue. (**B**) Phase II metabolism polymorphisms (*NAT2* and *UGT1A6*) were related to (R0–R1) LM resection, diarrhoea and fatigue. (**C**) Phase 3 metabolism (*SLC15A2*, *SLC01B3*, *SLC22A1* and *ABCB1*) were influential on early response, diarrhoea and neutropenia.

DISCUSSION

VKORC1 and *NAT2* SNPs were identified for the first time, as critically influencing the main efficacy end points in patients receiving HAI chemotherapy jointly with i.v. cetuximab for liver metastases from *KRAS* wild-type colorectal cancer. The translational study population involved 81.2% of the patients registered from nine European cancer centres (Levi *et al*, 2016). Ninety-five SNPs were found out of the 207 regions in 34 drug metabolism genes, which had been selected in the Sequenom ADME panel. Sixteen SNPs in 10 genes displayed statistically significant relations with efficacy and/or toxicity, using stringent selection methodology, according to best practices for case/control association studies (Clarke *et al*, 2011). Preprocessing steps involved the removal of monomorphic alleles, samples with low

detection efficiency and SNPs not passing minor allele frequency and HWE test thresholds. Linkage disequilibrium analysis and genotypic association test were then performed, and multiple testing correction tests were applied to select significant SNPs (Clarke *et al*, 2011; Robinson *et al*, 2011).

Single-nucleotide polymorphisms in the promoter region of *VKORC1* (rs9923231) and its related intron (rs9934438) were significantly associated with both early and objective responses, while SNPs in the promoter region also correlated with overall survival. The promoter SNP-related differences were large, with respective early responders and 4-year survivors in 4.5% and 0% of the 21 heterozygous patients, as compared to 50% and 46% of the 8 homozygous T/T patients. Intermediate rates were found for both end points in C/C patients (30% and 18%, respectively). The *VKORC1* gene, located on chromosome 16, encodes for an enzymatic protein responsible for both the reduction of vitamin K 2,3-epoxide to the activated form, and the γ-carboxylation of several coagulation factors. *VKORC1* is the major pharmacodynamics target of warfarin anticoagulant therapy, with the determination of SNPs at rs9923231 and rs9934438 being recommended for warfarin dose adjustment (Wang *et al*, 2008; Owen *et al*, 2010). *VKORC1*-dependent γ-carboxylated proteins are also involved in bone formation (Johnson *et al*, 1991; Coutu *et al*, 2008), signal transduction (Nakano *et al*, 1997), antioxidation and lipid synthesis (Mukai *et al*, 1992; Fredericks *et al*, 2013), and androgen receptor regulation (Tew *et al*, 2017). The relevance of *VKORC1* is currently emerging for prostate cancer, while recent reports support its role for cellular proliferation, reactive oxygen species production and apoptosis (Di *et al*, 2017).

Two SNPs in the *NAT2* gene (rs1041983 and rs1801280) were associated with macroscopically complete liver metastases resection following effective triplet HAI and i.v. cetuximab. The *NAT2* gene, located on chromosome 8p22, encodes the enzyme involved in the acetylation of xenobiotics. Polymorphisms in the *NAT2* gene influence the slow *vs* fast acetylator status of individuals (McDonagh *et al*, 2014). The combination of both SNP genotyping used here displayed similar sensitivity and specificity as the conventional 7-SNP genotyping of *NAT2* for the determination of the acetylator phenotype (Selinski *et al*, 2011; Suarez-Kurtz *et al*, 2016). Here the SNP-related differences were large, with R0–R1 LM resections occurring in 50% of the heterozygous C/T genotype as compared to 14.7% of the homozygous ones (C/C or T/T). To the best of our knowledge, no relation has been reported as yet regarding *VKORC1* or *NAT2* SNPs and efficacy or toxicity outcomes in cancer patients. Thus, the OPTILIV protocol could represent a preferred treatment option for those patients with both initially non-resectable LM-CRC, and constitutive SNPs at *VKORC1* rs9923231 T/T, at *NAT2* rs1801280 T/T and rs1041983 C/T, and/or *ABCB1* rs1045642 T/T and rs20132582 T/T. Indeed, such 'optimal' association of SNPs was found in an OPTILIV patient apparently cured from 27 synchronous liver metastases, after a disease-free and treatment-free interval of 6.5 years (Figure 2 in Levi *et al.* 2016).

In contrast, SNPs in the other eight ADME genes have already been associated to various clinical end points in patients on chemotherapy. This was notably the case of *ABCB1*, whose SNPs at rs1045642 and rs2032582 were here associated with PFS, while those at rs1045642 were also associated with severe neutropenia. This suggested an important role of constitutive SNPs at this latter gene locus for chemotherapy detoxification both in host and in cancer cells. The SNPs in *ABCB1* have been associated with toxic events in patients receiving irinotecan-based chemotherapy or capecitabine (Cortejoso *et al*, 2013; Garcia-Gonzalez *et al*, 2015) while a prognostic effect of ABCB1 variant (rs2032582) was reported for the overall survival of patients receiving FOLFIRI for metastatic colorectal cancer (De Mattia *et al*, 2013). In our study, the patients with T/T genotype in rs104642 had both worst neutropenia and best PFS. The same genotype was further associated with highest oxaliplatin and cetuximab exposure in the subset of patients undergoing pharmacokinetics determinations, while receiving chronomodulated triplet HAI. Severe neutropenia on FOLFOX has been shown to be a reliable prognostic indicator of PFS and OS in colorectal cancer patients (Innominato *et al*, 2011; Kasi *et al*, 2016). In addition, SNPs in five other ADME genes (*CYP2C9*, *CYP2E1*, *UGT1A6*, *SLC01B3* and *SLC22A1*) only related to toxicity, suggesting their potential relevance for SNP-guided optimisation of tolerability without efficacy impairment.

In summary, the current pharmacogenetic investigation was carried out in previously treated patients receiving triplet HAI and i.v. cetuximab for unresectable LM from KRAS WT within a European prospective trial. The results emphasised critical and consistent roles for SNPs in *VKORC1* and *NAT2*, two genes whose relevance for outcomes in cancer patients had not been reported before. *ABCB1* polymorphism was further highlighted as a joint predictor of neutropenia and PFS. Although OPTILIV was a prospective and multicentric trial, and stringent criteria were used for reliably selecting the relevant SNPs, the current study involved a limited size population, hence requiring further confirmation. Indeed, polymorphisms in *VKORC1*, *NAT2* and *ABCB1* could help better tailor an aggressive liver-targeted medico-surgical strategy for LM from colorectal cancer.

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CONFLICT OF INTEREST

VB has consulted or advised for Merck Serono, Amgen, Sanofi-Aventis, Bayer and Prestizia; has received honoraria from Amgen, Merck Serono, Bayer, Roche, Novartis and Daiichi Sankyo; has received travel grants from Amgen, Merck Serono, Sanofi and Bayer; has received research funding from Merck Serono. JT has received honoraria from Merck, Amgen, Roche, Celgene, Sanofi, Lilly, Baxalta and Sirtex. RG has received travel grants Roche, Novartis, Ipsen, Sanofi and Lilly. RA has consulted or advised for Merck and Amgen; has received honoraria from Amgen, Merck, Sanofi and Astellas; and has received travel grants from Amgen, Merck, Sanofi and Astellas. MD has consulted or advised for Roche, Merck Serono, Amgen, Bohringer, Servier and Celgene; has received honoraria from Merck Serono, Roche, Amgen, Novartis, Lilly, Bayer, Ipsen, Servier and Celgene; has received travel grants from Roche, Merck Serono, Ipsen, Bayer and Celgene; and has received research funding from Merck Serono, Pfizer and Roche. His wife is Head of Business Unit Oncology of Sandoz. GM has consulted or advised for Merck, Pierre Fabre and Onxeo; has received honoraria from Merck, Roche, BMS, Novartis, Amgen and Pierre Fabre. The remaining authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

FL, AK, PI, MB, GM and AL designed the study; all co-authors collected the data; FL, AK, PI, CD and AL searched the literature; FL, AK, RS and CD analysed and interpreted the data; all co-authors were involved in the drafting or reviewing of report and approval of submitted manuscript.

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