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Evaluating the role of *ENOSF1* and *TYMS* variants as predictors in fluoropyrimidine-related toxicities: An IPD meta-analysis

Seid Hamzic[a,b], Dominic Kummer[a], Tanja K. Froehlich[a], Markus Joerger[c], Stefan Aebi[d], Claire Palles[e], Ian Thomlinson[f], Didier Meulendijks[g], Jan H.M. Schellens[h], Xandra García-González[i], Luis A. López-Fernández[i,j], Ursula Amstutz[a], Carlo R. Largiadèr[a,*]

[a] University Institute of Clinical Chemistry, University of Bern, Switzerland
[b] Graduate School for Cellular and Biomedical Sciences, University of Bern, Freiestrasse 1, CH-3012, Bern, Switzerland
[c] Department of Medical Oncology and Hematology, Cantonal Hospital St. Gallen, Rorschacherstrasse 95, CH-9007, St. Gallen, Switzerland
[d] Division of Medical Oncology, Cantonal Hospital Lucerne, Spitalstrasse, CH-6000, Lucerne 16, Switzerland
[e] Gastrointestinal Cancer Genetics, Institute of Cancer and Genomic Sciences, University of Birmingham, Edgbaston, Birmingham, UK
[f] Cancer Genetics and Evolution Laboratory, Institute of Cancer and Genomic Sciences, University of Birmingham, Edgbaston, Birmingham, UK
[g] Dutch Medicines Evaluation Board (CBG-MEB), Utrecht, the Netherlands
[h] Faculty of Science, Utrecht University, Utrecht, the Netherlands
[i] Pharmacy Department, Hospital General Universitario Gregorio Marañón, Gregorio Marañón Health Research Institute, Doctor Esquerdo 46, 28007, Madrid, Spain
[j] Spanish Clinical Research Network, Doctor Esquerdo 46, 28007, Madrid, Spain

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ABSTRACT

To assess the proposed associations of the c.742-227G > A (rs2612091) polymorphism within the Enolase Superfamily Member 1 gene (*ENOSF1*) and two variants in the adjacent Thymidylate Synthase gene (*TYMS*): the 5'VNTR 28bp-repeat (rs45445694) and 3'UTR 6bp-indel (rs11280056) with severe toxicity in fluoropyrimidine-treated cancer patients, we performed an individual patient data meta-analysis. Only studies investigating all three-abovementioned variants with fluoropyrimidine-related toxicities were considered for meta-analysis. Associations were tested individually for each study using multivariate regression. Meta-analysis was performed using a random-effects model. One-stage multivariate regressions including tests for independent SNP effects were applied to investigate individual effects of the variants. Multivariate haplotype regression analyses were performed on a pooled dataset to test multi-SNP effects. Of four studies including 2'067 patients, 1'912 were eligible for meta-analysis. All variants were exclusively associated with severe hand-foot-syndrome (HFS) (*TYMS* 2R: OR = 1.50, p = 0.0002; *TYMS* 6bp-ins: OR = 1.42 p = 0.0036; *ENOSF1* c.742-227G: OR = 1.64 p < 0.0001, per allele). We observed independent effects for *ENOSF1* c.742-227G > A and the *TYMS* 28bp-repeat: each toxicity-associated allele increased the risk for severe HFS (OR = 1.32 per allele, p < 0.0001). Patients homozygous for both variants were at the 3-fold higher risk for severe HFS compared to wild-type patients. Our results confirm an essential role for *ENOSF1* c.742-227G and *TYMS* 2R-alleles in the development of fluoropyrimidine-related HFS. This suggests an important function of these genes in the development of severe HFS. Furthermore, these variants might help stratify patients in studies investigating measures of HFS prevention.

1. Introduction

The two fluoropyrimidines (FP) 5-fluorouracil (5-FU) and the oral prodrug capecitabine (Cp) are widely used anti-cancer drugs. Several enzymatic steps are involved in their conversion to the main cytotoxic compound 5-fluoro-deoxyuridine monophosphate, which results in the inhibition of thymidylate synthase (TS) [1]. This inhibition leads to a nucleotide imbalance, and subsequently to cell death. Due to this rather unspecific mode of action, severe adverse effects are frequent and still present a major concern in FP-based chemotherapies [2]. Interestingly, the toxicity profiles of Cp and 5-FU differ considerably. Patients receiving Cp-based treatments are less likely to experience stomatitis and neutropenia, but are at 3-fold higher risk for developing severe hand-foot syndrome (HFS) [3]. A recently published phase three clinical trial

* Corresponding author.
E-mail address: carlo.largiader@insel.ch (C.R. Largiadèr).

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using Cp in patients with biliary tract cancer reported severe HFS as the most common toxicity (20 % frequency of grade 3 HFS). Furthermore, this study reported that around 5 % of the patients discontinued treatment exclusively due to HFS [4].

It is well established that the rate of degradation of 5-FU plays an important role in the development of FP-induced toxicity. Specifically, a reduced activity in the first and rate-limiting enzyme of the pyrimidine catabolic pathway, dihydropyrimidine dehydrogenase (DPD), increases the risk of developing severe FP toxicity through an accumulation of cytotoxic metabolites [5]. A recent prospective study showed that pre-therapeutic screening of deleterious DPD gene (*DPYD*) variants conferring reduced activity or complete enzyme deficiency is clinically useful for reducing the occurrence of severe toxicities in FP-treated patients [6]. Nevertheless, a majority of toxic effects remains unexplained.

The gene encoding for TS (*TYMS)* has been extensively investigated to assess potential biomarkers for FP treatment safety and effectiveness. Two variants in *TYMS*, the 5′UTR tandem 28bp-repeat (5′VNTR) (rs45445694) and the 3′UTR 6bp-indel (rs11280056), have been investigated by multiple groups [7]. Indeed, recent studies suggest a role for these variants in the development of FP-related toxicities. Two meta-analyses reported that patients carrying two 28bp repeats (2R) instead of three (3R) in the *TYMS* 5′ UTR have a higher toxicity risk [8,9]. Similarly, the *TYMS* 3′UTR 6bp-ins was also associated with increased FP-related toxicity in a meta-analysis [9]. However, the modest size of the effect of both of these variants did not support their clinical implementation as biomarkers for toxicity risk prediction.

A recent study reported that the G allele of a novel variant in the Enolase Superfamily Member 1 gene (*ENOSF1*), c.742-227G > A (rs2612091), which is in partial genetic linkage with the *TYMS* 6bp-indel and *TYMS* 28bp-repeat, was associated with global Cp-induced toxicity, and in particular with HFS [10]. Additionally, this variant seemed to explain the previously reported associations with the *TYMS* 6bp-ins and the *TYMS* 28bp 2R-allele [10]. This association of *ENOSF1* c.742-227G > A with HFS could be replicated, and another study further reported an association with overall survival (OS) and gastrointestinal toxicities [11,12]. The role of *ENOSF1* in FP-metabolism or mechanism of action is still not fully elucidated.

ENOSF1 was first described as reverse Thymidylate Synthase (*rTS*) and a *TYMS* antisense gene [13]. The *ENOSF1* and *TYMS* genes partially overlap on chromosome 18 and are transcribed in opposite directions. So far, three *ENOSF1* isoforms have been described. One of them was identified as a L-fuconate dehydratase. None of the isoforms showed a metabolic function in 5-FU metabolism [14]. However, *in vitro* studies suggested that *ENOSF1* might be involved in the regulation of *TYMS* at the protein and the RNA level and thus affect 5-FU treatment as a *TYMS* regulator [15]. In order to evaluate the roles of the partially linked variants: *ENOSF1* c.742-227G > A, *TYMS* 6bp-indel, and *TYMS* 28bp-repeat in the safety of FP-chemotherapy, we performed an individual patient data (IPD) level meta-analysis of all available studies investigating these three variants in relation to adverse effects from FP-chemotherapy. In addition, we genotyped the same candidate variants in our own cohort consisting of 144 Cp-treated and of 403 5-FU-treated patients.

2. Methods

2.1. Patients and sample collection

Between 2006 and 2013, blood samples of 547 consenting FP-treated patients were collected at the Bern University Hospital (Switzerland) and the Cantonal Hospital St. Gallen (Switzerland) described previously in more detail [16,17]. All 547 patients provided signed informed consent. Except for 15 subjects, all patients were of self-declared Caucasian origin. Patient characteristics, chemotherapies, and FP-associated toxicities within the first two cycles of chemotherapy were recorded and graded according to the Common Terminology Criteria for Adverse Events (CTCAE) v3.0 [18]. One patient had to be excluded from the initial cohort due potential confusion of sample identity. This cohort is referred as Hamzic et al. in the meta-analysis.

2.2. DNA sequencing and genotyping

Genomic DNA was extracted from EDTA blood samples as described previously [16]. For genotyping of candidate polymorphism *ENOSF1* c.742-227G > A (rs2612091), a validated TaqMan single nucleotide polymorphism (SNP) genotyping assay (Thermo Fisher Scientific, assay C_15908768_10) and a 7500 fast real-time polymerase chain reaction (PCR) system (Thermo Fisher Scientific) were used. To determine the variable number of tandem repeats in *TYMS*, the 5′UTR promoter region was amplified (primers: forward 5′-GTGGCTCCTGCGTTTCC CCC-3′, reverse 5′-GCTCCGAGCCGGCCACAGGCATG-3′) using a GC-rich PCR System (Roche Applied Science). The amplification resulted in an amplicon size of 242 bp (3 repeats – 3R) and 214 bp (2 repeats – 2R), respectively. PCR reactions were performed in GeneAmp 9800 Fast Thermal Cyclers or GeneAmp 9700 Thermal Cyclers (Thermo Fisher Scientific) with an initial denaturation step of 3 min at 96 °C, 45 cycles of 30 s at 96 °C, 30 s at 60 °C, and 45 s at 72 °C, followed by a final extension step of 10 min at 72 °C. To distinguish 2R and 3R genotypes, amplified fragments were separated on a 1.5 % agarose gel. For the analysis of the 6 bp ins-del polymorphism (c.*447_452del), a fragment of the 3′UTR was amplified using a fluorescently labeled primer (forward 5′-FAM – CCACGTACTTATAAAGAAGGTTGGTG-3′, reverse 5′-CAGAATGAACAAAGCGTGGACGAAT-3′). Amplifications were performed using the QIAGEN Multiplex PCR kit (Qiagen) and the same instrumentation as described for the *TYMS* promoter region. PCR reactions started with an initial denaturation step of 15 min at 95 °C, followed by 22 cycles of 30 s at 94 °C, 1 min 30 s at 56 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. A mixture of 0.55 µl GeneScan LIZ 600 Size Standard (Thermo Fisher Scientific) and 10.45 µl Hi-Di formamide (Thermo Fisher Scientific) was added to 1 µl of PCR product and denatured for 3 min at 95 °C. Denatured products were subsequently resolved on an ABI Prism 3130xl Genetic Analyzer (Thermo Fisher Scientific) and genotypes were determined using the GeneMapper software v.4.0 (Thermo Fisher Scientific).

2.3. Study inclusion and data gathering

For this meta-analysis we focused on published studies, which investigated the effects of *ENOSF1* c.742-227G > A (rs2612091), *TYMS* 6bp-indel (rs11280056), and *TYMS* 28bp-repeat (rs45445694) on FP-related toxicities. The criteria for inclusion were (i) that the study has genotyped all three candidate variants and (ii) investigated them for association with severe FP-related toxicities. Furthermore, all studies had to be conducted according to the Helsinki Declaration and individual toxicities had to be graded according CTCAE v3 or v4. Only prospectively collected cohorts were considered. Literature search was last conducted on April 11 2019. We used the search term "*ENOSF1* AND *TYMS* AND (Fluorouracil OR 5-FU OR Capecitabine)" on Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) and Embase (https://www.embase.com/#search). In total, the search yielded 17 hits (Supplementary Fig. 1). Since rs2612091 in *ENOSF1* was the most recently reported variant, it was the limiting factor for study inclusion. We performed additional searches with the keywords "*ENOSF1*" and "rs2612091" used separately, which yielded 69 hits in total. However, this search did not reveal in any additional studies meeting the inclusion criteria. In total, three studies met the criteria to be included in our meta-analysis. We contacted the authors of these studies, who all agreed to participate in this analysis [10–12]. The different study cohorts are referred to as follows: Rosmarin et al. [10], Meulendijks et al. [12], Garcia-Gonzalez et al. [11], and Hamzic et al. (our cohort). We collected individual level data on genotypes for rs2612091,

rs11280056, rs45445694, age, sex, type of cancer, tumor stage, treatment setting, regimen including concomitant chemotherapy, number of treatment cycles, *DPYD* genotypes (c. 1905+1G > A (rs3918290, *DPYD**2A), c. 1679T > G (rs55886062, *DPYD**13), c. 2846A > T (rs67376798) and c.1129-5923C > A (rs75017182, c.1236G > A/HapB3)), and individual FP-related toxicities. All studies provided individual toxicity levels, except from Meulendijks et al. where data for each individual patient was only available in a pre-coded format, i.e. categorized into severe grade ≥ 3 or ≤ 2 toxicities. The authors of Garcia-Gonzalez et al. provided unpublished data from 81 additional patients of their study, leading to an extended cohort of 320 patients. Only patients with complete genetic data were included into the analyses. This meta-analysis does not include new human data that requires ethical approval. All included cohorts were collected in accordance with the Declaration of Helsinki (10–12,16,17).

2.4. Association testing and meta-analysis

For this meta-analysis, we chose a two-stage approach. In the first stage, each study was tested individually for association of genetic variants with severe FP-induced toxicity (grade ≥ 3) using multivariate logistic regression and odds ratios as summary measure. Sex, age, 5-FU/Cp, and *DPYD* risk variant carrier status were included as co-factors for every cohort. *DPYD* risk variant carrier status was coded with a binary variable ('risk'), where carriers of one of the four clinically relevant DPD dysfunctional variants (c.1905+1G > A (rs3918290), c.1679T > G (rs55886062), c.2846A > T (rs67376798) and c.1129-5923C > A (rs75017182, c.1236G > A/HapB3) were classified as 'risk variant carrier' and all others as' non-carriers'. Furthermore, concomitant chemotherapy was included as co-factor depending on their association with severe overall toxicity in the individual cohort. Overall toxicity was defined as the highest toxicity grade observed in any toxicity category in any cycle. All individual toxicity types were tested using the same multivariate regression model. Publication bias was assessed for all investigated individual outcomes by funnel plots and Egger's test. Due to the different therapy schemes and duration of toxicity recording, we performed the meta-analysis using the DerSimonian-Laird random-effects model with the R-package "metafor" [19,20]. Statistical heterogeneity was assessed with I^2-statistic and Cochrane's Q-test. All variants were tested assuming an additive allele model. Multivariate regression including all variants in the pooled dataset was performed in one stage with the "rms" R-package. For one-stage analyses of the pooled dataset, we introduced an additional categorical co-variable into the model in order to account for cohort-dependent effects. Furthermore, we included every concomitant chemotherapy, which was significantly associated in the individual studies with overall toxicity ('cisplatin, 'oxaliplatin' and 'bevacizumab') as co-variables into the one-stage regression. In order to analyze independent effects of the investigated candidate variants, we performed multivariate analysis using the "–independent-effects"- function implemented in PLINK version v1.07 on the pooled dataset in one-stage including the abovementioned covariates. This function compares alleles that have a similar haplotypic background in order to find independent SNP effects. A cut-off p-value of $p < 0.05$ was chosen, and no adjustment for multiple testing was performed due to significant linkage among all three candidate variants. The PRISMA-IPD checklist was used as guideline for the writing of the manuscript [21].

2.5. Post-hoc analysis

In order to test the effect of the *TYMS* variants independent of the *ENOSF1* c.742-227G > A genotype in the pooled dataset, a *post-hoc* analysis was performed including only patients homozygous for the *ENOSF1* c.742-227A allele (n = 567), using the same multivariate regression as mentioned above.

2.6. Haplotype analysis

For haplotype inference, we used two functions of the hap.stats package in R, which allows for ambiguous haplotypes where linkage is not complete [22–24]. The posterior probabilities are used to compute the score with the "haplo.score"-function and are used as weights for the regression coefficient in the "haplo.glm" function-based analysis. The merged dataset (n = 1'912) was used for this analysis, which was performed in one stage as mentioned above in order to increase the power for rare haplotypes. Genetic linkage between the three variants was calculated with the "genetics" package in R.

2.7. Allele-score analysis

Analyses using an allele-score combining genotypes of *TYMS* 28bp-repeat and *ENOSF1* c.742-227A > G were performed based on a pooled dataset including individual patient data of all study cohorts (n = 1'912). The allele-score of a patient was calculated as the total number of toxicity-associated alleles across the two variants. For each toxicity associated allele copy at any of the two loci, the score increases by 1. For example, a patient heterozygous for a toxicity-associated allele for one of the variants received a score of one. Patients homozygous for all of the toxicity-associated alleles at both loci received the maximum score of four, whereas patients carrying no toxicity-associated allele received the minimum score of zero.

3. Results

3.1. Study inclusion and characteristics

We identified three studies in the literature search, which investigated the effects of *ENOSF1* c.742-227G > A (rs2612091), *TYMS* 6bp-indel (rs11280056), and *TYMS* 28bp-repeat (rs45445694) in relation to FP-chemotherapies and were suitable for inclusion in the meta-analysis (Table 1) search results and strategy are described in Supplementary Fig. 1. Only one study investigating *ENOSF1* c.742-227G > A with FP-related toxicities in 62 Cp-treated patients was not included into this analysis, since it did not include any of the *TYMS* variants [25]. From all included studies, we received individual patient data. Toxicity data from the study of Meulendijks et al. was provided pre-coded for each individual patient, i.e. categorized into in severe grade ≥ 3 and grade ≤ 2 toxicities. In total, combined with the unpublished data from our own cohort referred to as Hamzic et al., we included data from four studies and 2'067 FP-treated patients. Only patients with no missing genotypes for any of the *TYMS*, *ENOSF1,* and *DPYD* loci were included in the analyses, which led to the exclusion of 154 patients. One additional patient was excluded due potential sample mix-up. Two individual patients were observed to carry a very rare *TYMS* 28bp 4R allele. In order to simplify the analysis, these patients were also classified as carriers of the 3R allele in this study.

Of the remaining 1'912 patients included in the analysis, 1'511 were treated with Cp mono- or combination therapy, whereas the remaining 402 were treated with infusional 5-FU mono- or combination chemotherapy. We observed a high heterogeneity in the frequency of the observed toxicity types among the studies, most likely due to differences in therapy schemes and number of cycles considered for toxicity recording (Table 1).

3.2. Meta-analysis

The only variant associated with overall toxicity was the *TYMS* 6bp-ins (OR = 1.21 p = 0.0215), where carriers of the 6bp-ins allele experienced higher overall toxicity rates (20.3 % of del/del-carriers vs. 28.3 % of ins/del carriers vs. 30.3 % ins/ins-carriers; Supplementary Fig. 2). No significant publication bias was observed for *TYMS* 6bp-indel and overall toxicity (Egger's test p = 0.15). However, for the

Table 1
Characteristics of included study cohorts. GIN: Gastrointestinal Toxicites, HEM: Hematological Toxicities, HFS: Hand-foot-syndrome, OVE: Overall Toxicity, *DPYD* risk: Combined frequency of *DPYD* risk alleles (c.1905 + 1G > A (rs3918290), c.1679T > G (rs55886062), c.2846A > T (rs67376798) and c.1129-5923C > A (rs75017182, c.1236G > A/HapB3). *Compared to the published cohort, 81 additional patients were provided.**bevacizumab, cetuximab, panitimumab and others.

						Allele frequencies in %							
Cohort	Age	Female %	FP-treatment	Patients	Treatment regimen	GIN	HEM	HFS	OVE	*DPYD* risk	*TYMS* 28bp-Repeat *(2R)*	*TYMS* 6bp-indel *(ins)*	*ENOSF1* c.742-227A > G *(G)*
Garcia-Gonzalez et al.*	67 (29–87)	48 %	Capecitabine	320	Cp mono, XELOX, XELIRI +- Abs**	16 %	10 %	8 %	29 %	1 %	43 %	67 %	44 %
Hamzic et al.	63 (28–99)	40 %	Capecitabine 5-FU	545	5FU (+- LV), FOLFOX, FOLFIRI, FOLFOXIRI, Cp mono, XELOX, XELIRI + other +- Abs**	9 %	9 %	2 %	17 %	3 %	46 %	69 %	48 %
Meulendijks et al.	58 (26–77)	27 %	Capecitabine	184	DOC, ECC, B-DOC, B-DOCT	14 %	32 %	3 %	44 %	6 %	51 %	70 %	46 %
Rosmarin et al.	64 (27–85)	42 %	Capecitabine	863	Cp mono or Cp + bevacizumab	13 %	1 %	23 %	32 %	3 %	48 %	68 %	45 %

other two variants, we observed significant publication bias with overall toxicity (*TYMS* 28bp-repeat and *ENOSF1* c.742-227G > A, Egger's test: p = 0.01 and p < 0.01, respectively). This result was driven by the study of Rosmarin et al., which reported the highest frequency of severe HFS for their cohort (Table 1). When excluding this study, no significant association of *TYMS* 6bp-indel with overall toxicity was observed (Supplementary Fig. 2). All three variants were significantly associated with severe (grade 3) HFS (*TYMS* 28bp 2R-allele: OR = 1.50, p = 0.0002; *TYMS* 6bp-ins: OR = 1.41 p = 0.0036; *ENOSF1* c.742-227G: OR = 1.64 p < 0.0001; Fig. 1). When excluding the study of Rosmarin et al. from the analysis, all three variants showed ORs of similar magnitudes and the *TYMS* 28bp 2R-variant (OR = 1.71, p = 0.0287) and *TYMS* 6bp-ins (OR = 1.81, p = 0.0423) remained significantly associated with severe HFS (*ENOSF1* c.742-227G: OR = 1.58 p = 0.0604). No significant study heterogeneity and no publication bias was observed with HFS as an outcome (Fig. 1, Supplementary Fig. 3). Individual frequencies of severe HFS for each individual cohort are reported in the Supplementary Table 1. No significant associations with any other individual toxicity type was detected (Supplementary Fig. 2).

In the multivariate logistic regression of the pooled dataset (n = 1'912) including all three loci in the regression model, only *ENOSF1* c.742-227G > A remained significantly associated with severe HFS (*TYMS* 28bp 2R-allele: p = 0.2603, OR: 1.17 [0.89–1.52]; *TYMS* 6bp-ins: p = 0.6336, OR 1.07 [0.80–1.43]; *ENOSF1* c.742-227G: p = 0.0176, OR: 1.45 [1.07–1.97]). However, when we performed multivariate analyses applying the "–independent-effects"- function implemented in PLINK version v1.07, we observed significant independent effects for the *TYMS* 28bp 2R- and *ENOSF1* c.742-227G-alleles (*TYMS* 28bp 2R-allele: $p_{overall}$ = 0.0439; *ENOSF1* c.742-227G: $p_{overall}$ = 0.0015). No statistically significant independent effect could be observed for the *TYMS* 6bp-ins (*TYMS* 6bp-ins: $p_{overall}$ = 0.6280) (Supplementary Table 2).

Based on this result, we carried out a *post-hoc* analysis for assessing the individual associations of *TYMS* 2R and *TYMS* 6bp-ins with HFS-toxicity excluding all patients carrying the *ENOSF1* c.742-227G variant. In this sub-cohort of patients, who are homozygous for the protective A-allele of *ENOSF1* c.742-227G > A (n = 567), we observed a significant increase in HFS for each 2R allele copy (OR: 1.82 95 %CI: [1.13–2.92], p = 0.0132), and a non-significant increase for the *TYMS* 6bp-ins copy (OR: 1.23 95 %CI: [0.78–1.92], p = 0.3723) in a multivariate logistic regression analysis.

In summary, these results thus suggest independent effects for the *TYMS* 28bp 2R-allele and for the *ENOSF1* c.742-227G-allele, whereas, the association of the *TYMS* 6bp-ins may be driven by the partial linkage with the other two variants.

3.3. Haplotype analyses

We observed strong linkage between *ENOSF1* c.742-227G > A and *TYMS* 6bp-indel (D' = 0.90-0.95, R^2 = 0.31-0.39), and moderate linkage among these two alleles and the *TYMS* 28bp-repeat (*ENOSF1* c.742-227G > A -*TYMS* 28bp-repeat; D' = 0.58-0.74, R^2 = 0.32-0.46 and *TYMS* 6bp-indel-*TYMS* 28bp-repeat; D' = 0.43-0.61, R^2 = 0.07-0.14) in all cohorts (Supplementary Table 3).

The most frequent haplotype (H1: 36.7 %), which consists of all toxicity-associated variants at the three loci, showed the strongest association with increased HFS risk (Table 2). The second most frequent haplotype carrying none of the toxicity-associated alleles (H2: 24.3 %) was significantly associated with reduced HFS-toxicity risk as was haplotype H3 (H3: 19.9 %) carrying only the 6bp-ins allele. Haplotypes composed of *TYMS* 6bp-ins and the *TYMS* 2R or the *ENOSF1* G-allele (H4: 8.2 % and H6: 3.5 %) were associated with severe (grade 3) HFS, when compared to the protective haplotype H2 (Haplo.GLM function). No other haplotype with only one toxicity-associated allele was significantly associated with increased severe (grade 3) HFS (H3: 19.9 %

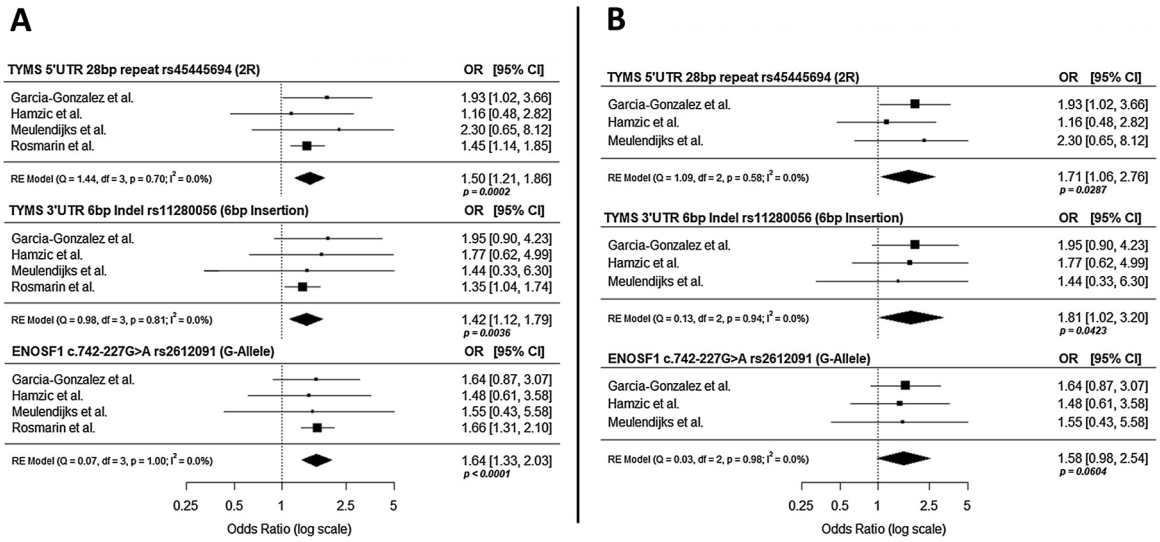

Fig. 1. Forest plots of meta-analyses of *ENOSF1* and *TYMS* polymorphisms (*TYMS* 28bp-repeat, *TYMS* 6bp-indel, and *ENOSF1* c.742-227A > G) associated with severe HFS (grade 3). (A) Including all studies (B) Meta-analysis with exclusion of the cohort from Rosmarin et al. The toxicity-associated alleles are shown in brackets. The square-size is proportional to the contribution of each study to the association. Horizontal lines and overall diamond width represent the 95 % CI of observed odds ratios (OR).

Table 2
Association of three-SNP haplotypes with severe (grade 3) HFS. OR: Odds Ratios, Toxicity associated candidate SNPs are in bold and underlined. Significant p-values are in bold. Haplotypes were calculated using a multivariate additive model.

	Haplotypes				Haplo.Score			Haplo.GLM	
	TYMS 28bp-repeat	*TYMS* 6bp-indel	*ENOSF1* c.742-227A > G	Haplotype Freq.	Hap-Score	P-value	Sim P-value	P-value	OR [95 % CI]
H1	**2R**	**Ins**	**G**	36.72 %	3.496	**0.0005**	**0.0002**	**< 0.0001**	**1.75 [1.31–2.35]**
H2	3R	Del	A	24.31 %	−3.102	**0.0019**	**0.0017**	*Base Haplotype*	
H3	3R	**Ins**	A	19.92 %	−2.514	**0.0119**	**0.0119**	0.766	0.96 [0.65–1.39]
H4	3R	**Ins**	**G**	8.20 %	1.565	0.1176	0.1177	**< 0.0001**	**2.26 [1.45–3.52]**
H5	**2R**	Del	A	6.50 %	−0.193	0.8467	0.8460	0.344	1.33 [0.74–2.38]
H6	**2R**	**Ins**	A	3.47 %	1.712	0.0868	0.0836	**0.018**	**2.06 [1.12–3.79]**
H7	3R	Del	**G**	0.58 %	−0.796	0.4258	0.4393	0.644	0.63 [0.07–5.38]
H8	**2R**	Del	**G**	0.31 %	−0.110	0.9123	0.8989	0.704	1.60 [0.14–17.9]

and H5: 6.5 %, H7: 0.58 % and H8: 0.31 %). These results further support the associations of *ENOSF1* c.742-227 G and *TYMS* 2R-allele with severe HFS, but not for the *TYMS* 6bp-ins allele.

3.4. Allele-score analysis

We used an additive allele-score to investigate the effect of multiple allele carriers of *ENOSF1* c.742-227G > A and *TYMS* 28bp-repeat on the risk for developing severe grade 3 HFS in the pooled dataset (n = 1'912). We observed a 3-fold increased risk of severe HFS in patients homozygous for the two toxicity-associated alleles, compared to patients carrying none of the two variants (frequency of severe HFS 18.4 % vs. 6.1 %) (Fig. 2a). A multivariate regression in the pooled dataset showed a significant increase of severe HFS with increasing allele-scores (OR: 1.32 95 %CI: [1.18–1.49], per allele, p < 0.0001). In comparison, we observed a frequency of 17.3 % of severe HFS in *DPYD* risk variant carriers and 12.3 % in non-carriers, respectively. The frequency of patients homozygous for *ENOSF1* G- and *TYMS* 2R-alleles was 13.9 %, whereas the frequency of patients carrying none of the two alleles was 19.0 % (Fig. 2a). The overall frequencies of severe HFS (grade 3) according to the individual SNPs in the pooled dataset are shown in Fig. 2b

4. Discussion

This is the first meta-analysis evaluating the role of the recently reported *ENOSF1* variant c.742-227G > A with FP-related toxicities. We included all published studies, which investigated *ENOSF1* c.742-227G > A together with the *TYMS* 28bp-repeat and *TYMS* 6bp-indel variants for association of this variant with fluoropyrimidine related toxicities. Including individual patient data allowed us to apply the same genetic- and toxicity models for each study, improving the comparability among studies in the meta-analysis.

Our results strongly support that *ENOSF1* c.742-227G > A is an important pharmacogenetic marker for FP-related HFS. Furthermore, our findings are in line with previous studies reporting significant associations of *TYMS* 28bp 2R-allele and *TYMS* 6bp-ins with FP-related toxicities [8,10]. To follow up on these associations, this study also investigated if *ENOSF1* c.742-227G > A is the underlying causal variant for this association, i.e. if the associations of both *TYMS* variants were solely a result of genetic linkage with *ENOSF1* c.742-227G > A, as previously suggested [10]. While the results from the multivariate regression analysis including all variants support this hypothesis, our analyses for independent SNP-effects performed in PLINK indicate independent effects for both *ENOSF1* c.742-227G > A and the *TYMS* 28bp-repeat. The observation that the *TYMS* 28bp-repeat was not associated with HFS in the multivariate regression may have resulted from a bias introduced by multi-collinearity among the presumably

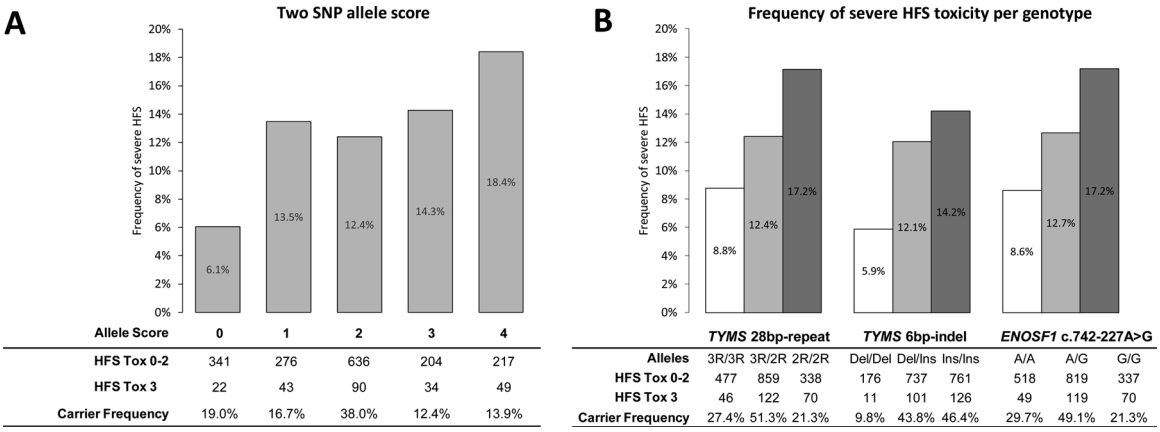

Fig. 2. Two SNP allele-score and frequency of severe (grade 3) HFS according to the genotypes. (A) Two SNP allele score with *ENOSF1* c.742-227A > G and *TYMS* 28bp-repeat in the total cohort (n = 1'912). The score ranges from zero (no toxicity associated alleles) to four (homozygous for *ENOSF1* c.742-227A > G and *TYMS* 28bp-repeat). (B) Frequencies of severe (grade 3) HFS according to individual genotypes of *ENOSF1* and *TYMS* polymorphisms (*TYMS* 28bp-repeat, *TYMS* 6bp-indel, and *ENOSF1* c.742-227A > G) in the total cohort (n = 1'912).

independent variables in the model. In other words, the observed partial genetic linkage among the variants, resulting in correlation of the corresponding variables, may have masked the effect of the *TYMS* 28bp-repeat in this analysis. Finally, the independent effect of the *TYMS* 28bp 2R-allele is supported by its association with HFS in a *post-hoc* analysis including only patients with identical *ENOSF1* genotype (c.742-227AA).

In contrast, our analyses (*e.g.* using the "–independent-effects"-function implemented in PLINK or the haplotype analyses) provided no indication that the observed associations of the *TYMS* 6bp-ins with overall toxicity and HFS in the two-stage meta-analysis represent or tag an additional independent genetic effect. The variant was also not found to be significantly associated with HFS in the *post-hoc* analysis excluding the *ENOSF1* c.742-227 G effect. The association of *TYMS* 6bp-ins with HFS observed in the meta-analysis can be caused through a hitch-hiking effect with the *ENOSF1* c.742-227G-allele - *i.e.* through the strong linkage between the two loci (D' = 0.90-0.95, R^2 = 0.31-0.39).

Due to the linkage among the three variants, it is very difficult to estimate the effect sizes of each individual SNP based on the current data. At present, we may only conclude that there is a strong indication for at least two independent genetic effects contributing to increased FP-related HFS-risk. Furthermore, we cannot exclude the possibility that the studied variants are tagging other, yet unknown causal variants. Therefore, we assumed an additive model for the allele-score and the haplotype analyses. The results confirmed that carriers of multiple associated alleles were at higher risk for toxicity, compared to patients with none or only one toxicity-associated allele. Our results are mostly relevant for Cp-treated patients, since 5-FU-treated patients show a much lower incidence of HFS [7]. Even though HFS is not a life-threatening toxicity, a recent phase three clinical trial reported severe HFS as a major factor leading to chemotherapy cessation upon patient's choice [4]. Cp chemotherapy may thus be generally improved if patients with a high risk of developing severe HFS could be identified prior to starting therapy. Furthermore, there have been efforts to prevent HFS in Cp treated patients: Several studies investigated using topical urea/lactic acid, pyridoxine and cyclooxygenase-2 inhibitors for HFS prevention in a meta-analysis with mixed results [26]. Another recent study observed that patients following non-drug related therapeutic measures developed significantly less HFS [27]. High-risk patients could also potentially benefit from infusional 5-FU therapy to reduce the risk of severe HFS. We recommend genotyping *ENOSF1* c.742-227A > G and *TYMS* 28-bp repeat in upcoming studies investigating HFS preventing therapeutic measures for better stratification of the patients potentially benefiting from such therapies. Furthermore, the diagnostic potential of these candidate variants as pharmacogenetic markers needs further evaluation in an independent prospective study.

Our data suggest an important role of genetic variation in both *TYMS* and *ENOSF1* in the development of HFS. However, the underlying biological mechanisms inducing HFS are unknown, as well as the biological function of *ENOSF1*. For *ENOSF1,* its role in 5-FU metabolism is not known and a direct function of this gene in the metabolism of 5-FU seems unlikely according to the study of Wicheleki et al. [14]. Nevertheless, several *in-vitro* experiments suggest that *ENOSF1* may act as a regulator of *TYMS* at the mRNA and the protein level [15]. However, further studies investigating the biological mechanism underlying HFS and the function of *ENOSF1* in FP-chemotherapy are needed.

Previously, *ENOSF1* c.742-227G > A has been reported as an eQTL for *ENOSF1* but not for *TYMS* [10]. However, more recent data from GTEx Portal (v7) indicated that *ENOSF1* c.742-227G > A is an eQTL for both, *ENOSF1* and *TYMS* in many different tissues (Supplementary Fig. 4). Therefore, a direct regulatory effect of the c.742-227G > A variant on *TYMS* expression cannot be excluded. The *TYMS* 6bp-indel is also an eQTL for both genes according GTEx Portal (v7) [28]. Interestingly, the direction of the effect on mRNA levels is highly tissue dependent for both variants. Additionally, *ENOSF1* c.742-227G > A and *TYMS* 6bp-indel are in significant linkage with many other variants, which are strong eQTLs for both genes in various tissues (data not shown). Taken together, these findings could explain some of the discrepancies between *in vitro* and clinical association studies for *TYMS* 6bp-indel: It was suggested that the 6bp-deletion in the 3'UTR leads to less stable mRNA and therefore less expression [29]. This is in contrast to our and other meta-analyses, where the 6bp-ins was associated with more FP-related toxicities, which suggest lower expression of *TYMS* for the 6bp-ins allele [9]. However, our analysis suggests that the association of the *TYMS* 6bp-ins with toxicity is driven by genetic linkage with the other two candidate variants and is likely not an independent factor for the development of FP-related toxicities. Therefore, any direct biological function of the *TYMS* 6bp-indel variant remains to be clarified.

The current literature is more coherent with respect to our results obtained for the *TYMS* 28bp-repeat: In line with our and other studies, the 2R allele was associated with an increased risk of FP-related toxicities [8,9,30,31]. Furthermore, the 2R allele has been associated with a better response and less intra-tumoral expression of *TYMS* [31,32]. Less expression of *TYMS* with the 2R-allele was also observed *in-vitro* [33] [30,31]. All these data support that the *TYMS* 28bp repeat is an independent and direct causal factor affecting *TYMS* expression. However, the promoter structure in *TYMS* is actually more complicated: There is also a G > C polymorphism found in this genomic region. The

C-allele was reported to disrupt a USF-1 binding site causing lower *TYMS* expression *in-vitro* [34,35]. This SNP can be present in all 28bp-repeat genotypes and a recent study showed that carriers of the 2R-allele with the C-variant (2RC/2RC, 2RG/2RC, 3RC/2RC) were at higher risk for Cp-related toxicities compared to non-carriers [36]. However, these variants are rather rare and the information of this sub-classification was not available for all cohorts, which is a limitation of this study. No data from GTEx Portal could be retrieved for *TYMS* 28bp-repeat, most likely due to the complexity of genotyping this variant. Another important limitation of this study is that haplotypes were inferred by statistical methods, which can be problematic when only partial linkage is present. This limitation could be overcome with phased genotyping, which can be performed with new long-read sequencing technologies in order to determine haplotype structure unambiguously in each individual patient sample by direct observation. This would give more insights in the genetic structure underlying the observed associations and may help identify potential other causal variants. For example, a recent study reported additional novel variants in the *ENOSF1/TYMS* region, which are associated with FP-induced toxicities [37]. Therefore, it is possible that this genetic region harbors further important markers for toxicity prediction. More studies investigating potential associations in this genetic region are thus needed.

5. Conclusion

In conclusion, our results strongly support the recently reported independent association of the variant *ENOSF1* c.742-227G > A variant with severe HFS in FP-treated patients. Furthermore, the *TYMS* 28bp-repeat was also independently associated with severe HFS. On the other hand, the association of the *TYMS* 6bp-ins seems to be driven by genetic linkage with the other two candidate variants. Based on the current data, it is impossible to infer exact effect sizes of each individual variant due to the considerable linkage among all three polymorphisms. However, we may conclude that carriers of multiple copies of the associated variants are at higher risk for severe HFS as compared to patients carrying none or only one variant and that the studied genomic region very likely harbors more than one independent genetic risk factor for fluoropyrimidine-related HFS.

Genotyping these variants could help to improve patient stratification in upcoming studies investigating HFS preventing therapeutic measures. In order to verify the diagnostic potential of these variants, prospective studies are needed.

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Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

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