Genetic polymorphism in *ATIC* is associated with effectiveness and toxicity of pemetrexed in non-small-cell lung cancer

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ABSTRACT

Patients with advanced non-small-cell lung cancer who are treated with pemetrexed display a wide variation in clinical response and toxicity. In this prospective, multicentre cohort study, we investigated the association with treatment effectiveness and toxicity of 10 polymorphisms in nine candidate genes, covering the folate pathway (*MTHFR*), cell transport (*SLC19A1/ABCC2/ABCC4*), intracellular metabolism (*FPGS/GGH*) and target enzymes (*TYMS/DHFR/ATIC*) of pemetrexed. Adjusted for sex, ECOG performance score and disease stage, the association between *ATIC* (rs12995526) and overall survival (HR 1.59, 95% CI 1.06 to 2.39) was significant. Regarding toxicity, this *ATIC* polymorphism was significantly associated with severe laboratory (p=0.014) and clinical (p=0.016) chemotherapy-related adverse events, severe neutropenia (p=0.007) and all-grade diarrhoea (p=0.034) in multivariable analyses.

BACKGROUND

Pemetrexed is widely used in the treatment of advanced non-small-cell lung cancer (NSCLC) as first-line treatment in combination with a platinum agent, and recently also immunotherapy, second-line therapy and maintenance treatment.[1] Pemetrexed shows a substantial variation in clinical effectiveness and toxicity, which cannot be predicted for individual patients. Importantly, toxicity is related to the pharmacokinetic (PK) parameters of pemetrexed, which have a wide interpatient variability.[2]

Here, we aimed to investigate whether polymorphisms of genes associated with the pharmacodynamics (figure 1), which cover the folate pathway (*MTHFR*), cell transport (*SLC19A1/ABCC2/ABCC4*), intracellular metabolism (*FPGS/GGH*) and target molecules (*TYMS/DHFR/ATIC*) of pemetrexed, are associated with clinical effectiveness and toxicity of pemetrexed in a large cohort of patients exposed to this drug. Additionally, we explored the relationship of these pharmacogenetic single nucleotide polymorphisms (SNPs) with the PK of pemetrexed.

MATERIALS AND METHODS

Pharmacogenetic data were available from the 'PEmetrexed and biomaRkerS: an observatiONAL study' (PERSONAL), a prospective multicentre cohort study in the Netherlands. Adult patients with locally advanced or metastatic (stage IIIB/IV) non-squamous NSCLC receiving per standard of care (online supplemental methods) platinum-combined pemetrexed therapy as first-line treatment, followed by maintenance pemetrexed if indicated, or pemetrexed monotherapy as second-line treatment, were recruited from October 2012 until November 2014. The Institutional Review Board of the Erasmus University Medical Center approved this study and all patients provided written informed consent.

Adverse events (AEs) were registered weekly during the entire treatment period and graded according to the National Cancer Institute - Common Terminology Criteria for Adverse Events (NCI-CTCAE) V.4.03 (grade ≥3 was marked as severe toxicity).[3] Clinical effectiveness endpoints were overall survival (OS), progression-free survival (PFS) and best tumour response according to the Response Evaluation Criteria In Solid Tumors (RECIST) V.1.1.

DNA isolation and genotyping are described in the online supplemental methods.

Within the *TYMS* gene, two polymorphisms were selected and combined as one genotype, resulting in a high-expression (3RG/3RG), intermediate expression (3RG/3RC, 2R/3RG) and low expression genotype (2R/2R, 2R/3RC, 3RC/3RC).[4]

For details on SNP selection and statistical analyses, see online supplemental methods. We used our recently developed population-PK model as a base model for the current pharmacokinetic/pharmacodynamic analyses,[5] in which SNPs were included as covariables on pemetrexed clearance. Cox regression analysis was applied in treatment-naïve patients to test the association between polymorphisms and OS/PFS. Adjustment for sex, Eastern Cooperative Oncology Group (ECOG) performance score and disease stage was performed. Polymorphisms were tested against toxicity endpoints using cause-specific Cox regression analyses. If the patient died before completion of four cycles of chemotherapy, censoring for death was performed to take this into account as a competing risk.[6] In univariable analyses, correction for multiple testing was applied using the false discovery rate (FDR) Benjamini-Hochberg procedure (significance level $p<0.1$). A two-sided $p<0.05$ was regarded as significant in multivariable analyses.

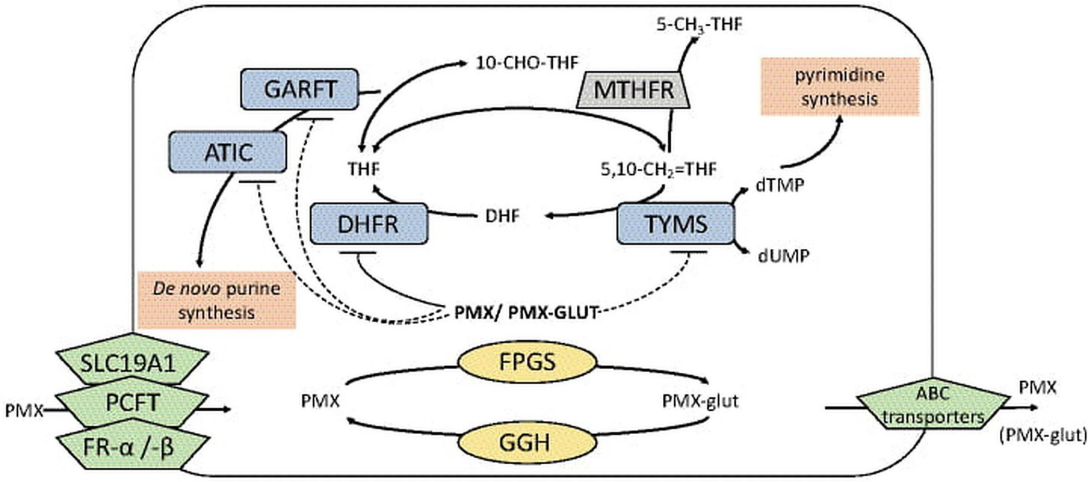

Figure 1 Important proteins involved in the working mechanism of pemetrexed. Green boxes: enzymes involved in the cell transport of pemetrexed. The most important import transporter reduced folate carrier (RFC) is encoded by SLC19A1. Pemetrexed and its polyglutamates are excreted from the cell via ABC transporters, but polyglutamates to a lesser extent. Yellow boxes: FPGS is responsible for the polyglutamylation of pemetrexed and GGH for the deglutamylation. Blue boxes: TYMS, DHFR, GARFT and ATIC are the target enzymes of pemetrexed. The dashed lines represent the increased inhibitory ability of the pemetrexed polyglutamates compared with pemetrexed. Grey box: MTHFR has a major impact on the regulation of the folic acid pathway due to conversion of 5,10-methylenetetrahydrofolate to 5-methyl-THF, which is the methyl donor for methylation of dUMP to dTMP for de novo dTMP synthesis. PMX, pemetrexed; PMX-glut, pemetrexed polyglutamates; dTMP, deoxythymidylate; dUMP, deoxyuridine monophosphate.

RESULTS

One hundred sixty-three patients were recruited, of whom 161 (99%) provided blood samples for pharmacogenetic analysis (online supplemental table 1). Half of the patients were male with a mean age of 63.3±9. Most patients had metastatic NSCLC (87%) and received first-line platinum-combined chemotherapy (91%). Treatment-naïve patients (n=147) had a median OS of 7.7 months and PFS of 4.7 months. Forty-four patients (30%) continued with pemetrexed maintenance after induction treatment. The results of the pharmacogenetic analyses are demonstrated in table 1.

None of the polymorphisms were associated with tumour response (online supplemental table 2). In the univariable analyses, only the *ATIC* polymorphism (rs12995526) was significantly correlated with OS after FDR correction (table 2). Adjusted for sex, disease stage and ECOG performance score, the association between *ATIC* and OS remained (HR 1.59, 95% CI 1.06 to 2.39). Patients with a homozygous variant genotype (CC) had a significantly shorter OS compared with patients with CT/TT genotypes (6.2 months, 95% CI 3.4 to 9.0 vs 9.0 months, 95% CI 5.6 to 12.3, p=0.012), but this association was not found for PFS (online supplemental figure 1).

Detailed information about frequencies of treatment-related AEs and univariable analyses between SNP polymorphisms and toxicity are provided in online supplemental tables 3 and 4. In multivariable analyses (table 2), the homozygous variant genotype of *ATIC* was significantly associated with a 1.9-fold higher risk of severe laboratory and clinical AEs, a 2.0-fold higher risk of developing diarrhoea and a 2.3-fold higher risk of severe neutropenia. Univariably, the CC genotype of *ATIC* was also associated with experiencing severe fatigue and severe anaemia, and having at least one mutant *SLC19A1* allele was associated with an almost sevenfold lower risk of experiencing severe anorexia. These associations could not be tested multivariably due to a too low number of events.

Treatment-naïve patients with the CC genotype of *ATIC* had more dose reductions (OR 4.16, 95% CI 1.59 to 10.93, p=0.004), which was not significantly associated with OS. They continued less often with maintenance treatment than patients with the CT/TT genotypes (20% vs 33%, p=0.09). Receiving maintenance therapy was associated with improved OS (HR 0.59, 95% CI 0.40 to 0.87, p=0.01). Patients who experienced severe clinical toxicities during induction treatment received less often maintenance treatment than patients without these toxicities (19% vs 39%, p=0.01).

No significant associations were observed between the selected SNPs and pemetrexed clearance in the pharmacokinetic/pharmacodynamic analyses (online supplemental table 5).

DISCUSSION

We have found new associations between a genetic polymorphism in a gene encoding for pemetrexed target enzyme ATIC and overall survival, as well as pemetrexed-induced (severe) toxicity. None of the investigated polymorphisms could explain a part of the interpatient variability in pemetrexed PK.

Patients with homozygous variant *ATIC* alleles had a 1.6-fold higher risk of death and they experienced approximately two times more treatment-related toxicities than patients with CT/TT genotypes. Patients with this genotype also had an approximately four times higher risk of receiving dose reductions due to toxicity and they received less maintenance treatments. According to our data, a lower OS in patients with homozygous variant alleles of *ATIC* may be explained by more severe treatment-related toxicity leading to dose reductions and less maintenance treatment. However, a decreased OS is not mediated by increased tumour growth due to increased purine synthesis or decreased activations of AMP-activated protein kinase, as *ATIC* genotype was not associated with tumour response and/or PFS. The effect of the intronic polymorphism rs12995526 on *ATIC* functionality has not been clarified yet. Recently, Zhang *et al* did find an association between the CC genotype of the same *ATIC* polymorphism and worse tumour response, but no survival analyses were performed and therefore we cannot easily compare these findings with our results.[7] However, the specific ethnic Han Chinese population together with the high number

Table 1 Investigated single nucleotide polymorphisms in the total cohort (n=161)

Gene	rsID	Variant	Assay ID		WT (%)	HET (%)	HVAR (%)	MAF	HWE
SLC19A1	rs1051298	c.*746C>T	C__26854602_10		58 (36.0)	81 (50.3)	22 (13.7)	39%	0.45
GGH	rs3780126	c.109+1307C>T	C__26361922_20		65 (40.4)	74 (46.0)	22 (13.7)	37%	0.90
FPGS	rs1544105	g.2572C>T	C___8342611_10		53 (32.9)	79 (49.1)	29 (18.0)	43%	0.95
ABCC2	rs717620	c.-24C>T	C___2814642_10		100 (62.1)	56 (34.8)	5 (3.1)	20%	0.39
ABCC4	rs7317112	c.75-23516T>C	C__29165801_20		73 (45.3)	80 (49.7)	8 (5.0)	30%	0.02†
ATIC	rs12995526	c.815-102T>C	Assay-by-Design		39 (24.2)	79 (49.1)	43 (26.7)	49%	0.82
DHFR	rs1650697	c.-473T>C	C__27863089_10		90 (55.9)	57 (35.4)	14 (8.7)	26%	0.26
MTHFR	rs1801133	c.665C>T	C___1202883_20		72 (44.7)	77 (47.8)	12 (7.5)	31%	0.16
*TYMS**	rs45445694	5′UTR TSER*2/TSER*3	*See Reference*[4]	Genotypes				25%	0.34
	rs183205964	c.-86G>C		2R/2R	37 (23.0)				
				2R/3RC	42 (26.1)				
				2R/3RG	28 (17.4)				
				3RC/3RC	13 (8.1)				
				3RC/3RG	24 (14.9)				
				3RG/3RG	13 (8.1)				
				Undetermined	4 (2.5)				

**TYMS* was categorised into a high expression genotype (3RG/3RG), an intermediate expression genotype (3RG/3RC, 2R/3RG) and a low expression genotype (2R/2R, 2R/3RC, 3RC/3RC) depending on the 5′-UTR variable number tandem repeat (VNTR) polymorphism and the C/G polymorphism within the third VNTR.
†Since *ABCC4* 2168T>C was not in Hardy-Weinberg equilibrium, this SNP was excluded from all further analyses.
HET, heterozygous; HVAR, homozygous variant; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency; rsID, reference SNP ID number; TSER, thymidylate synthase enhancer region; WT, wild type.

of never smokers (66%), in contrast to our population (never smokers 3%), probably results in genetically different tumours, which might alter tumour behaviour and response to treatment.

Although this could not be confirmed multivariably, the CT+TT genotype of *SLC19A1* was univariably associated with a sevenfold lower risk of severe anorexia. Adjei *et al* showed an association between CT+TT genotype and a shorter PFS/OS.[8] Although the influence of the SNP, located in the 3′-UTR region, on gene functionality and expression is unknown, one could speculate that the CT+TT genotype may lead to a decreased influx of pemetrexed into the cell and thereby lower toxicity and less effectiveness.

To our knowledge, this is the largest NSCLC patient population treated with first-line pemetrexed in which pharmacogenetic analyses have been performed. However, a limitation of our study is still the relatively small sample size, which may have led to missed (weaker) associations between SNP genotypes and treatment outcomes due to a lack of statistical power.

A recent shift in the treatment paradigm of advanced NSCLC has led to the common use of platinum and pemetrexed plus pembrolizumab induction regimen followed by pemetrexed plus pembrolizumab maintenance. Although the survival has improved with the combination treatment of chemo/immunotherapy,[9] the

Table 2 Association between polymorphisms and PFS and OS in treatment-naïve patients and toxicity in all patients

					Univariable			Multivariable*	
Endpoint	**SNP**	**rsID**	**Model**	**Genotype**	**HR (95% CI)**	**P value**	**Adjusted P value†**	**HR (95% CI)**	**P value**
Clinical effectiveness (n=147)									
OS	*ATIC*	rs12995526	Recessive	CC vs CT+TT	1.65 (1.11 to 2.45)	0.010	0.080	1.59 (1.06 to 2.39)	0.025
	TYMS	rs45445694 rs183205964	High vs intermediate +low	3G/3G vs 3G/3C, 2R/3G+2R/2R, 2R/3C, 3C/3C	1.79 (0.97 to 3.28)	0.062	0.244		
PFS	*FPGS*	rs1544105	Dominant	TT +CT vs CC	0.65 (0.46 to 0.93)	0.012	0.096	0.72 (0.50 to 1.05)	0.084
Toxicity (n=161)									
Clinical									
Any event, grade 3/4	*ATIC*	rs12995526	Recessive	CC vs CT+TT	1.80 (1.10 to 2.96)	0.012	0.096	1.86 (1.12 to 3.07)	0.016
Diarrhoea, all grade	*ATIC*	rs12995526	Recessive	CC vs CT+TT	2.01 (1.07 to 3.78)	0.012	0.096	1.99 (1.05 to 3.77)	0.034
Fatigue, severe	*ATIC*	rs12995526	Recessive	CC vs CT+TT	3.33 (1.47 to 7.56)	0.004	0.032‡		
Anorexia, Severe	*SLC19A1*	rs1051298	Dominant	TT+CT vs CC	0.15 (0.03 to 0.72)	0.008	0.064‡		
Laboratory									
Any event, grade 3/4	*ATIC*	rs12995526	Recessive	CC vs CT+TT	1.89 (1.15 to 3.10)	0.011	0.088	1.87 (1.14 to 3.09)	0.014
Neutropenia, grade 3/4	*ATIC*	rs12995526	Recessive	CC vs CT+TT	2.24 (1.25 to 4.03)	0.007	0.056	2.25 (1.25 to 4.06)	0.007
Anaemia, severe	*ATIC*	rs12995526	Recessive	CC vs CT+TT	4.38 (1.67 to 11.52)	0.003	0.024‡		

*For clinical effectiveness endpoints adjusted for sex, disease stage and ECOG performance score. For toxicity endpoints adjusted for sex and age.
†Significant p<0.1 after false discovery rate correction.
‡Only tested univariably due to the number of events.
OS, overall survival; PFS, progression-free survival; rsID, reference SNP ID number.

combination also leads to more severe toxicity and withdrawal of induction treatment.[9 10] To date, germline genetic aberrations in genes involved in the PD-1 pathway have no clinical utility in predicting PD-1 inhibitor-associated toxicities.[11] But now, polymorphism analysis of *ATIC* (rs12995526) could provide valuable information on which patients are more vulnerable to severe pemetrexed-related toxicities. Our suggestion that decreased survival in patients with the CC genotype of *ATIC* may be a result of increased toxicity is alarming, but warrants further validation.

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Contributors SV, RM, SK, JA and BHS were involved in study design. SV, CvdL, NvW and RC were involved in recruiting patients and collecting data. Genetic analyses were performed by RvS and NvD and pharmacogenetic analyses by SV and SK. Statistical analyses were performed by SV and BHS. All authors were involved in data interpretation and manuscript writing.

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Competing interests JA reports personal fees and non-financial support from Eli Lilly, outside the submitted work. In addition, JA has a patent allogenic tumor cell lysate licensed to Amphera pending, and a patent for combination immunotherapy in cancer and biomarker for immunotherapy pending; BHS reports grants from ZonMw during the conduct of the study.

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Supplementary Methods

Rationale for candidate gene and SNP selection

Although the precise reasons for this interindividual variability have not yet been discovered, several pharmacokinetic processes of pemetrexed and its mechanism of action are already well known (Figure 1). Pemetrexed is primarily eliminated via the kidneys, and hence pemetrexed clearance and total exposure are associated with renal (dys)function [1,2]. Uptake into the cells is regulated by different membrane transporters, i.e. proton-coupled folate transporter (PCFT), folate receptors α and β, and reduced folate carrier (RFC), while ATP-binding cassette transporters (ABC) of the multidrug resistance protein family ABCC1-5 are primarily responsible for the cellular efflux of (anti-)folates [3,4]. Intracellularly, pemetrexed undergoes rapid polyglutamation facilitated by folylpoly-γ-glutamate synthetase (FPGS) and γ-glutamyl hydrolase (GGH) is involved in the reverse process of deglutamation [3]. The formation of polyglutamates is thought to be a major determinant of its antitumor activity as polyglutamates are no substrates for most efflux ABCC transporters, except ABCC5, and therefore are longer retained intracellularly. Moreover, polyglutamates have a stronger affinity for the target enzymes of pemetrexed [5]. Thymidylate synthetase (TYMS) is the main target enzyme of pemetrexed and results in disturbed *de novo* thymidine production needed for DNA synthesis. By binding to its secondary target enzymes glycinamide ribunecleotide formyltransferase (GARFT) and 5-aminoimadizaole-4-carboxamide ribonucleotide formyltransferase (ATIC) *de novo* purine synthesis is also inhibited, while binding to dihydrofolate reductase (DHFR) results in a diminished active tetrahydrofolate pool needed for purine and thymidine synthesis. ATIC may also play a role in cell growth and proliferation by inhibition of the mammalian target of rapamycin (mTOR) pathway [6]. Knockdown of ATIC by pemetrexed leads to an endogenous increase in 5-aminoimidazole-4-

carboxamide-1-β-D-ribonucleotide (AICAR), which activates AMP-activated protein kinase (AMPK) and inhibits its downstream pathway mTOR, and thereby ultimately leads to a decrease in cell proliferation and an increase in cell apoptosis [7,8].

Another potential determinant of pemetrexed activity is 5,10-methylenetetrahydrofolate reductase (MTHFR), which is an important regulator of the folic acid pathway [9]. It is both involved in DNA synthesis and methylation. Different levels of activity of all these different proteins, for example due to genetic variations, may lead to altered exposure and sensitivity to pemetrexed. In our study, we aimed to investigate whether polymorphisms of genes (Figure 1), which encode for or regulate these enzymes, are associated with clinical effectiveness and toxicity of pemetrexed in a large cohort of patients exposed to this drug.

Based on its role in the working mechanism of pemetrexed, earlier findings with regard to the relation of polymorphisms and clinical outcomes and a minor allele frequency of >10% in the European subpopulation of the 1000 Genome project using LDpop [10], we selected SNPs of the above mentioned genes.

The polymorphism *746C>T of *SLC19A1*, encoding the major entrance transporter RFC, has been associated with progression-free and overall survival (PFS/OS) in a small group of NSCLC patients treated with the combination pemetrexed-bevacizumab and in a mixed NSCLC/mesothelioma cohort [11,12]. This polymorphism is located in the 3'-UTR region of *SLC19A1*. The SNP *ABCC2* -24C>T, has been reported to lower the expression of the protein [13], which theoretically leads to intracellular accumulation of pemetrexed (polyglutamates) and might explain the better objective tumor response in patients with the -24CC polymorphism and the increased gastrointestinal toxicity observed with the TT polymorphism in patients treated with pemetrexed [14,15]. In patients with acute lymphocytic leukemia receiving treatment with methotrexate, closely resembling the

mechanism of cell transport of pemetrexed, the wildtype variant of SNP *ABCC4* 75-23516T>C was associated with a higher risk of mucositis [16]. The polymorphism is located in intron 1 of the *ABCC4* gene, its role has not been clarified yet. With regard to the metabolizing enzymes, there is evidence that alterations in FPGS and GGH function may alter the cellular retentions of (anti)folates [17,18]. *GGH* intronic polymorphism 109+1307C>T was associated with worse median overall survival and less hematological toxicity [11]. The wildtype variant of *FPGS* 2572C>T correlated with a higher protein expression of FPGS and higher response rate [19]. *TYMS* mRNA expression is regulated by different polymorphisms, among others various number of 28-base-pair tandem repeats (VNRT) in 5' UTR enhancer region of the *TYMS* gene, and a SNP -86G>C inside this second tandem repeat [20]. Patients with a low expression genotype had a more favorable clinical response to pemetrexed, while they experienced more hematological toxicities [21–25]. The T missense variant of the *MTHFR* 677C>T has been associated with reduced enzyme activity, and thus the carrying TT genotype would be expected to lead to a favorable clinical response. Reports on *MTHFR* 677TT genotype showed contradictory results with regard to OS/PFS [12,26,27]. The *DHFR* variant c.-473T>C is located in the 5'-promotor region of the gene and wildtype T allele forms part of a promoter region haplotype that is reported to upregulate *DHFR* expression. Carrying the TT genotype was associated with increased risk of adverse events [12]. With regard to the *ATIC* polymorphism c.815-102T>C, its relation with treatment effectiveness outcomes are contradictory. Woo et al. found that patients with the CC genotype had a better tumor response and overall survival, while Zhang et al. observed a worse tumor response in patients with the CC genotype without having performed survival analysis [14,28]. The effect of the intronic *ATIC* SNP on protein expression or functionality is not clear.

Materials and methods

Standard of care platinum-combined pemetrexed chemotherapy

Patients received platinum-combined pemetrexed chemotherapy or pemetrexed monotherapy treatment as first-line or second-line treatment per standard of care for a maximum of 4 cycles. Pemetrexed was dosed at 500 mg/m^2 and cisplatin at 75 mg/m^2. Carboplatin dosage was calculated using the Calvert formula with a target AUC of 5 or 6. Dose adjustments (i.e. reductions) at the start of subsequent courses of therapy were based on nadir counts (neutrophils, platelets) or maximal non-hematologic toxicity from the preceding cycle of therapy. Patients were recommended to continue with pemetrexed maintenance therapy if they had no progressive disease, no intolerable toxicities and underwent no sequential radiotherapy or surgery.

DNA isolation and genotyping

Four hundred microliters of whole-blood specimens collected in EDTA tubes were extracted on the MAGNAPure Compact (Roche Diagnostics GmbH, Germany) using the Total Nucleic Acid Isolation Kit I (Roche Diagnostics GmbH, Germany) and a final elution volume of 200 µl.

Taqman genotyping

The genotyping of *SLC19A1* 746C>T (rs1015298), *GGH* 6699G>A (rs3780126), *FPGS* 2572C>T (rs1544105), *ABCC2* -24C>T (rs717620), *ABCC4* 2168T>C (rs7317112), *ATIC* 815-102T>C (rs12995526), *MTHFR* 677C>T (rs1801133), *TYMS* VNTR polymorphism (rs45445694) and c.-86G>C (rs183205964) was performed using TaqMan 5'-nuclease analyses (ThermoFisher, Carlsbad, CA, USA). The assay IDs are listed in Table 1. Each assay consisted of two allele-specific minor groove binding (MGB) probes, labeled with the

fluorescent dyes VIC and FAM. Polymerase chain reactions (PCRs) were performed in a reaction volume of 10 µl, containing assay-specific primers, allele-specific Taqman MGB probes (Applied Biosystems), Abgene Absolute QPCR ROx Mix (Thermo Scientific, Life Technologies Europe BV, Bleiswijk, The Netherlands) and genomic DNA (20 ng).

Statistical analyses

The distribution of genotypes was tested for Hardy-Weinberg equilibrium (HWE) using the chi-squared test. Since *ABCC4* 75-23516T>C was not in HWE in our cohort (Table 1), this SNP was excluded from all further analyses.

With regard to toxicity endpoints, AEs were selected if they occurred in >10% of the patients. If an adverse event was already present in an equal or higher degree before start of treatment, it was not considered as an event. Adverse events were considered treatment-related if defined as possibly, probably or definitely related by the investigator. For both clinical effectiveness and toxicity end points, multivariable analysis was only performed in case of approximately 10 or more events per assessed variable in order to avoid bias of the regression coefficients. The selected polymorphisms were fitted and the most appropriate model was selected from four models: dominant, recessive, additive model and a multiplicative model [29].

With a sample size of patients treated with first-line pemetrexed n=147 and event rate (death) of 92% observed in our study, we were able to detect a hazard ratio of ≥ 2.0 (or ≤ 0.5) at a two-sided significance level of 0.025 (α=0.05) between two genotype groups with a power of 0.8 or higher, if the proportion of the dominant or recessive genotype group was ≥ 0.14. This is the case for all SNPs, except for the recessive genotypes of *MTHFR* (MAF 31%, n=12 (8.2%)), *ABCC2* (MAF 20%, n=5 (3.4%)), DHFR (MAF 26%, n=13 (8.8%)) and the high-expression genotype vs other of *TYMS* (MAF 25%, high-expression genotype n=12 (8.2%)).

For these genotypes the power of detection of HR ≥ 2.0 (or ≤ 0.5) was 0.35 (*ABCC2*), 0.6 (*MTHFR* and *TYMS*) and 0.64 (*DHFR*).

Statistical analyses were performed with the use of SPSS, version 24.0 (IBM Corporation, Armonk, NY).

Population pharmacokinetic model

The PK data were described by a two-compartment model (population estimate (% standard error of the estimate) in terms of pemetrexed clearance CL (4.58L/h (3.1%)), central volume of distribution V_c (15.9L (3.3%)), peripheral volume of distribution V_p (21.6L (5.0%)) and intercompartmental clearance (Q; 0.05L/h (4.7%)) [30]. Despite a reduction of approximately 20% in between-patient variability of pemetrexed clearance after inclusion of covariable estimated glomerular filtration rate (eGFR), still 16.7% (coefficient of variation) of the between-patient variability remained unexplained.

Genotypes encoding enzymes involved in the cell transport and polyglutamation of pemetrexed (SLC19A1, GGH, FPGS, ABCC2) were added to the previously developed population PK model and were included as dichotomous or ordinal covariables on pemetrexed clearance using the following equation:

$$CL = \theta_x * \frac{eGFR}{median}^{\theta_y} * (\theta_z)^{pg},$$

Where pg was scored '1' for patients of whom the genotype of interest was present and '0' for patients of whom the genotype was absent if the genotype was considered as a dichotomous variable (recessive or dominant genotype). If the genotype was included ordinally (additive genotype), pg was scored '0' for patients with the homozygous major allele genotype (wild-type), '1' for heterozygous patients and '2' for patients with the homozygous minor allele genotype (variant). θ_x is the typical parameter value for the homozygous major allele population, θ_y is the covariable effect size estimate of eGFR and θ_z

is the covariable effect size estimate of the SNP. First, the potential association of all SNPs was univariably tested. The threshold of this step was set at $p < 0.01$ (likelihood ratio test, Δ objective function value (OFV) > 6.64, degrees of freedom =1 or ΔOFV > 9.21, degrees of freedom =2). In the next step, all potentially related covariables were included in the full model. During a backward elimination procedure, covariables were removed one at a time from the full model again if the fit of the model did not decrease significantly ($p < 0.005$) tested using the likelihood ratio test (ΔOFV > 7.88, df =1 or (ΔOFV > 10.6, df =2).

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Supplementary table 1. Patient characteristics at baseline (n=161)

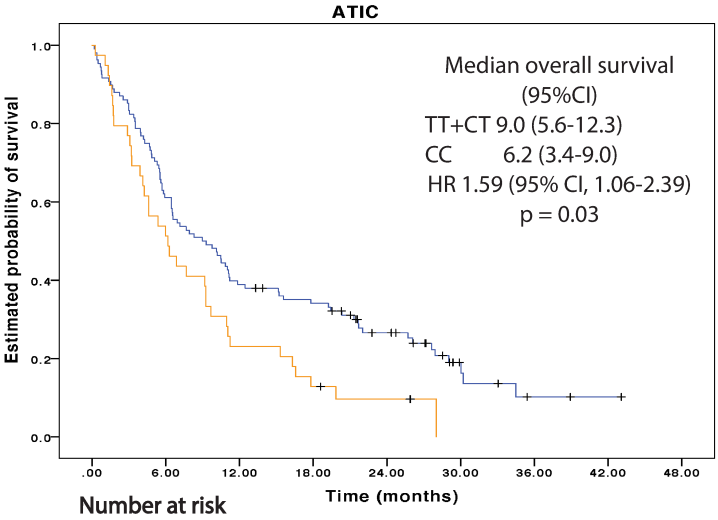
Characteristic	All patients (n = 161)
Age (yr), mean (SD)	63.3 (9.2)
Gender, male	82 (50.9)
Ethnicity, Caucasian	151 (93.8)
ECOG performance score	
0 or 1	140 (87.0)
≥ 2	19 (11.8)
Missing	2 (1.2)
Packyears	36.9 (33.9)
Never smokers	4 (2.5)
Type of tumor	
Adenocarcinoma	156 (96.9)
Large cell carcinoma	5 (3.1)
Cancer stage	
Locally advanced (IIIB)	21 (13.0)
Metastatic (IV)	140 (87.0)
Line of therapy	
First-line	147 (91.3)
Second-line	14 (8.7)
Combination therapy	
Cisplatin	99 (61.5)
Carboplatin	59 (36.6)
Monotherapy	3 (1.9)
Comorbidity	
Cardiovascular disease	68 (42.2)
COPD	23 (14.3)
Diabetes	22 (13.7)

Data are expressed as numbers (%) unless stated otherwise. Abbreviations: SD, standard deviation.

Supplementary Table 2. Associations of polymorphisms with objective tumor response (n=122)

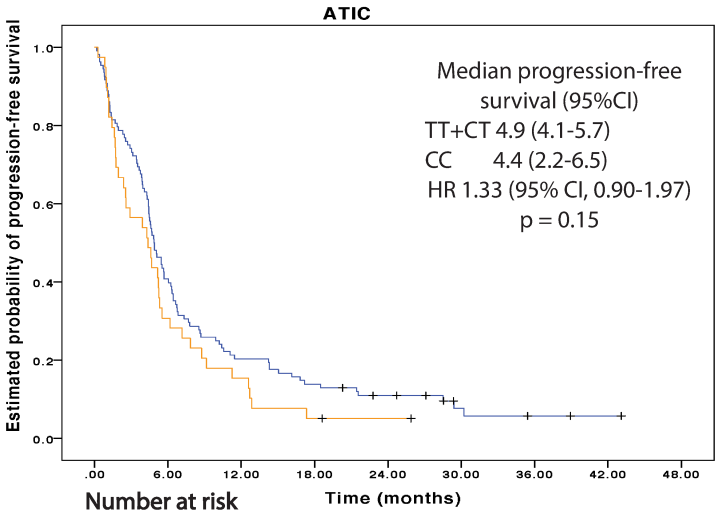
Endpoint	SNP	Model*	Genotype	Significance Test	Univariable OR (95% CI)	p-value
	ATIC	Recessive	CC vs CT + TT	Fisher's exact	0.90 (0.38 – 2.14)	0.810
	TYMS	High + intermediate vs low	3G/3G +3G/3C, 2R/3G vs 2R/2R, 2R/3C, 3C/3C	Chi-squared	0.68 (0.31 – 1.52)	0.351
	SLC19A1	Recessive	TT vs CT + CC	Fisher's Exact	3.31 (1.06 – 10.33)	0.063
ORR	*GGH*	Dominant	GA + AA vs GG	Chi-squared	1.86 (0.84 – 4.11)	0.12
	FPGS	Dominant	TT + CT vs CC	Chi-squared	0.62 (0.29 – 1.36)	0.235
	ABCC2	Dominant	TT + CT vs CC	Fisher's exact	1.23 (0.56 – 2.70)	0.610
	DHFR	Additive	TT → CT → CC	Logistic regression	1.12 (0.61 – 2.06)	0.720
	MTHFR	Recessive	TT vs CT + CC	Fisher's exact	0.91 (0.22 – 3.70)	0.892

*Best fitting model. Abbreviations: OR, odds ratio; ORR, objective response rate



Number at risk

TT+CT	108	66	42	35	22	7	2	1
CC	39	20	9	5	3	0	0	0



Number at risk

TT+CT	108	44	22	15	10	4	2	1
CC	39	12	6	2	1	0	0	0

Supplementary Table 3. Adverse events in patients with first- and second-line treatment (n=161)

	Frequency (%)	
Adverse event	All grades	Grade ≥ 3
Treatment-related[a]		
Any event	158 (98)	99 (62)
Clinical		
Any event	157 (98)	68 (42)
Fatigue	140 (87)	23 (14)
Nausea and vomiting	105 (65)	4 (2)
Anorexia	101 (63)	10 (6)
Oral mucositis/stomatitis	75 (47)	5 (3)
Constipation	66 (41)	2 (1)
Taste alteration	62 (39)	0
Dry skin	53 (33)	0
Dizziness	49 (30)	0
Neuropathy sensory	46 (29)	0
Dry eyes/watering eyes	44 (27)	0
Diarrhea	41 (25)	4 (2)
Infection with normal neutrophil count	38 (24)	19 (12)
Dysphagia	37 (23)	2 (1)
Rash	30 (19)	0
Weight loss	29 (18)	0
Alopecia	24 (15)	0
Abdominal distension	20 (12)	1 (1)
Pruritus	19 (12)	0
Laboratory		
Any event	154 (96)	68 (42)
Anemia	139 (86)	17 (11)
Decreased white cell count	106 (66)	27 (17)
Decreased neutrophil count	97 (60)	47 (29)
Alanine aminotransferase elevation	80 (50)	2 (1)
Decreased thrombocyte count	78 (48)	17 (11)
Alkaline phosphatase elevation	63 (39)	0
Aspartate aminotransferase elevation	68 (38)	2 (1)
Blood creatinine level elevation	54 (34)	2 (1)

Listed are adverse events that are reported in at least 10% of the patients. [a]Adverse events were scored as treatment-related if investigator defined relatedness as possibly, probably or definitely.

Supplementary Table 4. Association between SNPs and chemotherapy-related toxicity with p<0.1 in the univariable analysis

Endpoint	SNP	Model	Univariable	
			HR (95% CI)	p-value
Severe toxicity	*ATIC*	Recessive	1.54 (1.01-2.35)	0.045
Severe toxicity, laboratory	*ATIC*	Recessive	1.89 (1.15-3.10)	0.011†
	MTHFR	Recessive	2.09 (1.03-4.23)	0.041
Severe toxicity, clinical	*ATIC*	Recessive	1.80 (1.10-2.96)	0.012†
Clinical				
Anorexia, all grade	*FPGS*	Dominant	0.68 (0.45-1.02)	0.059
	ATIC	Dominant	0.68 (0.44-1.05)	0.078
Anorexia, severe	*SLC19A1*	Dominant	0.15 (0.03-0.72)	0.008†
Diarrhea, all grade	*ATIC*	Recessive	2.01 (1.07-3.78)	0.012†
	MTHFR	Dominant	0.52 (0.28-0.98)	0.042
Fatigue, all grade	*FPGS*	Dominant	0.72 (0.50-1.02)	0.061
Fatigue, severe	*ATIC*	Recessive	3.33 (1.47-7.56)	0.004†
	DHFR	Recessive	3.80 (1.41-10.27)	0.018
	SLC19A1	Recessive	2.36 (0.93-5.99)	0.071
	ABCC2	Dominant	2.28 (1.00-5.20)	0.050
Nausea and vomiting, all grade	*MTHFR*	Recessive	1.87 (1.00-3.51)	0.050
	SLC19A1	Recessive	1.59 (0.94-2.68)	0.082
Mucositis, all grade	*ATIC*	Recessive	0.61 (0.35-1.08)	0.090
Infection normal ANC, all grade	*ABCC2*	Recessive	3.83 (1.18-12.51)	0.026
Taste alteration, all grade	*ATIC*	Recessive	0.56 (0.29-1.08)	0.085
	GGH	Dominant	0.66 (0.40-1.08)	0.097
Constipation, all grade	*SLC19A1*	Dominant	0.60 (0.37-0.98)	0.042
	FPGS	Recessive	1.79 (1.02-3.16)	0.043
Dry and watery eyes, all grade	*ABCC2*	Dominant	0.50 (0.25-0.98)	0.044
Dysphagia, all grade	*ABCC2*	Recessive	3.35 (1.03-10.93)	0.045
	ATIC	Recessive	0.40 (0.16-1.02)	0.055
Alopecia, all grade	*ATIC*	Recessive	2.57 (1.15-5.75)	0.021
	TYMS	High + intermediate vs low	0.29 (0.10-0.86)	0.025
	MTHFR	Recessive	2.72 (0.93-7.98)	0.068
Abdominal distension, all grade	*MTHFR*	Recessive	3.31 (1.11-9.90)	0.032
	TYMS	High + intermediate vs low	0.35 (0.12-1.05)	0.062
	ABCC2	Dominant	2.13 (0.88-5.14)	0.093
Laboratory				
Anemia, severe	*ATIC*	Recessive	4.38 (1.67-11.52)	0.003†
	DHFR	Recessive	4.28 (1.39-13.15)	0.019
Leukopenia, severe	*DHFR*	Dominant	2.85 (1.28-6.36)	0.019
	ATIC	Recessive	2.01 (0.93-4.32)	0.076
Neutropenia, severe	*ATIC*	Recessive	2.24 (1.25-4.03)	0.007†
	DHFR	Dominant	1.63 (0.92-2.90)	0.094
	GGH	Dominant	1.70 (0.91-3.18)	0.097
Trombopenia, severe	*ATIC*	Recessive	2.63 (1.01-6.81)	0.047

† significant p<0.1 after false discovery rate correction. Abbreviations: HR, hazard ratio; CI, confidence interval

Supplementary Table 5. SNP covariable analysis on pemetrexed clearance in the full model using stepwise forward inclusion

Factor	Compared genotypes	OFV	ΔOFV*
Ordinal	**Structural base model + eGFR on CL and BSA on Vc**	**-743.8**	
SLC19A1	Mut/Mut vs Mut/WT vs WT/WT	-745.3	-1.6
GGH	Mut/Mut vs Mut/WT vs WT/WT	-745.1	-1.3
FPGS	Mut/Mut vs Mut/WT vs WT/WT	-744.8	-1.0
ABCC2	Mut/Mut vs Mut/WT vs WT/WT	-744.3	-0.6
Dichotomous			
		-743.8	
SLC19A1	WT/WT vs other	-744.3	-0.5
	Mut/Mut vs other	-744.4	-0.6
GGH	WT/WT vs other	-744.7	-0.9
	Mut/Mut vs other	-743.8	-0.0
FPGS	WT/WT vs other	-744.5	-0.8
	Mut/Mut vs other	-744.3	-0.5
ABCC2	WT/WT vs other	-743.8	-0.0
	Mut/Mut vs other	-744.2	-0.5

* To determine model fit, ΔOFV was used according to the likelihood ratio test following a chi-squared distribution. In the stepwise forward inclusion, the threshold for significant improvement of the model was set at $p < 0.01$ (dichotomous: ΔOFV > 6.64, df =1 or ordinal: ΔOFV > 9.21, df =2). In the backward elimination significant worsening of the model was set at $p < 0.005$ (Δ OFV > 7.88, df =1).

†Structural model: Two-compartment model in terms of pemetrexed clearance (CL), central distribution volume (Vc), intercompartimental clearance (Q) and peripheral volume of distribution (Vp), including between-patient variability on CL and proportional error model describing between-patient variability

Abbreviations: OFV, objective function value; CL, pemetrexed clearance; Vc, central volume of distribution; Vp, peripheral volume of distribution; BSA, body surface area; eGFR, estimated glomerular filtration rate; Mut, mutant; WT, wildtype