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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