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ORIGINAL ARTICLE

Characterization of Three *CYP2C19* Gene Variants by MassARRAY and Point of Care Techniques: Experience from a Czech Centre

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Abstract Distribution of cytochrome P450 2C19 enzyme gene (*CYP2C19*) variants affecting metabolism of clopidogrel was determined in 526 Czech patients after percutaneous coronary intervention using MassARRAY genotyping and compared to distribution in other populations of European descent. Fifty-three (10%) patients underwent parallel determination of *CYP2C19* genotypes from buccal swabs by a point of care technique with 100% concordance to the main genotyping platform. Observed *CYP2C19* genotypes were related to clopidogrel metabolism phenotypes and discussed in population context. Hereby, presented methodologies provide accurate *CYP2C19* genotyping results in a relatively short time of one up to 12 h and may, therefore, find the relevant place in the field of genotype-guided antiplatelet therapy.

Keywords CYP2C19 · Pharmacogenetics · Clopidogrel · Genotyping · Point of care

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Introduction

Pharmacogenetics (PGx)-study of variations in DNA sequences affecting drug response and/or toxicity (Yip et al. 2015)-has been gradually gaining its place in personalised patient care in various fields of medicine (Jani et al. 2015; Ortega et al. 2015; Petrek 2015; Tangamornsuksan et al. 2015). Clopidogrel, as the drug most commonly prescribed together with aspirin after percutaneous coronary intervention (PCI) to prevent atherothrombotic events in patients with acute coronary syndrome, has an important place among the drugs in which PGx tests proved to have adequate clinical validity. Available data, including those from meta-analyses, indicate that patients on clopidogrel carrying particular variants of the cytochrome P450 2C19 enzyme gene (*CYP2C19*) may be at an increased risk of adverse cardiovascular events compared with noncarriers (Hulot et al. 2010; Mega et al. 2010a, b; Sofi et al. 2011; Zabalza et al. 2012). Indeed, FDA black box warning suggests consideration of alternative therapy in patients identified as CYP2C19 poor metabolizers and notes that these patients could be identified by genotyping (US Food and Drug Administration 2010). Recommendations regarding *CYP2C19* genotyping in context of clopidogrel therapy have also been made by Clinical Pharmacogenetics Implementation Consortium (Scott et al. 2013). There have been a number of academic institutions which have implemented hospital-wide reporting of *CYP2C19* genotypes in their electronic medical records, also to facilitate health care provider decision on treatment by alternative platelet inhibitors, such as prasugrel and ticagrelor (Knauer et al. 2015). Recently, there also have been reports on cost-effectiveness of genotype-guided antiplatelet therapy (Jiang and You 2015; Kazi et al. 2014).

Wider application of PGx testing for the *CYP2C19* variants underlying altered responsiveness to clopidogrel has been hindered by time-lag between specimen sampling and results' reporting which, in usual routine, involves 3 or more days. However, in patients endangered by complications, the genotype knowledge is desirable at the time of prescription and any therapeutic adjustment should be decided in a very limited time frame (Knauer et al. 2015). In this context, we aimed at facilitating our genotyping by MassARRAY technology, by which turn-around-time of 12 h could be achieved, and additionally by utilisation of a commercial point of care (POC) genotyping technique producing result within 1 h. Our findings, including population data on *CYP2C19* variants relevant for clopidogrel metabolism in Czechs and other Europeans, are summarised in the following report.

Materials and Methods

Characteristics of the Study Group

Five hundred and twenty-six patients (138 females, 388 males) after PCI from University Hospital Olomouc underwent routine examination of *CYP2C19* in the period from March 2013 to February 2016. Briefly, a sample of peripheral blood was obtained for genotyping by MassARRAY® technology (AgenaBio, San Diego, USA). The mean patient age was 66 years and ranged 24–93 years. Randomly selected patient subset ($n = 53$, 14 females, 39 males) was tested in parallel using a POC technique-Spartan RX System (Spartan Bioscience Inc., Ottawa, Ontario, Canada) utilising a buccal swab specimen. The mean age in this patient subset was 57 years and it ranged 31–77 years. All subjects were unrelated subjects living in the Czech Republic, i.e., of Central European descent, speaking Czech language. Signed informed consent explaining the nature of the examination and containing approval with usage of the routine test results for scientific purpose was obtained before specimen collection from each subject.

CYP2C19 Genotyping by MassARRAY

Genomic DNA was isolated from 0.5 ml of peripheral blood drawn into K_3EDTA-coated tubes using Arrow automated extraction system (Isogen Life Science, De Meern, The Netherlands). Methodical details on MassARRAY procedure have previously been described, in general (Stacey et al. 2009), and also regarding our laboratory procedure (Kishore et al. 2015). In brief, the primer pairs (Assay design suite v2.0; https://agenacx.com/Tools) were multiplexed. Genotyping was performed using Sequenom MassARRAY platform integrating iPLEX® SBE (single base extension reaction) and MassARRAY® technology (Agena Bioscience, San Diego, CA, USA)-based MALDI-TOF MS assay. The assay consists of an initial locus-specific PCR amplification followed by SBE using mass-modified dideoxynucleotide terminators of an oligonucleotide primer that anneals immediately upstream of the target polymorphic site. The distinct mass of extended primer traces the alternative alleles using MassARRAY Typer 4.0.20. For quality control (QC) step, we determined data missing rate per individuals and missing rate per single-nucleotide polymorphism (SNP). In addition, for QC of SNP genotyping, positive and negative template control samples were included in each assay plate. Any assay found as positive in negative template control was removed from the study. The correctness of our *CYP2C19* genotyping has been repeatedly verified in three annual intervals (2013–2015) by successful participation in the external proficiency testing scheme "Molecular Genetics set 06" (775) provided by INSTAND, Düsseldorf, Germany.

CYP2C19 Genotyping Using POC Technique

In a subset of our patients ($n = 53$), *CYP2C19**2,*3 and *17 variants were determined by Spartan RX System (Spartan Bioscience Inc., Ottawa, Ontario, Canada)-a POC system based on processing of buccal swabs in a closed, semi-automated amplification system. According to the manufacturer, Spartan RX Analyser employs optical detection and subsequent automated analysis for evaluation of fluorescent signals from oligonucleotide probes binding to PCR amplicons and thus determinates the genotype; it consists of Spartan RX CYP2C19 Assay and Spartan RX Platform. Three sample collection kits, each for one of the three *CYP2C19* tested variants, were used to collect buccal swab samples. The collection kit, provided by the manufacturer, consists of a pouch containing a buccal swab and a reagent tube. The reagent tube contains chemicals for DNA extraction, PCR amplification, and fluorescent detection of the specific *CYP2C19* allele. Collection procedure and subsequent sample processing were performed according to Spartan RX System Operator's Manual (Document Number 01001920_1.5, 1–37). Briefly, before sample collection, patient rinsed the mouth by a small amount of water, then a buccal swab from the inside of patient's cheek was collected and samples were immediately processed in a nearby laboratory according to the manufacturer's instructions. Briefly, the reagents and buccal samples inside reagent tubes were mixed by taping on their bottom. Next, tubes were inserted into Spartan RX Analyser and in less than an hour, the test completed with automatic print-out of the results.

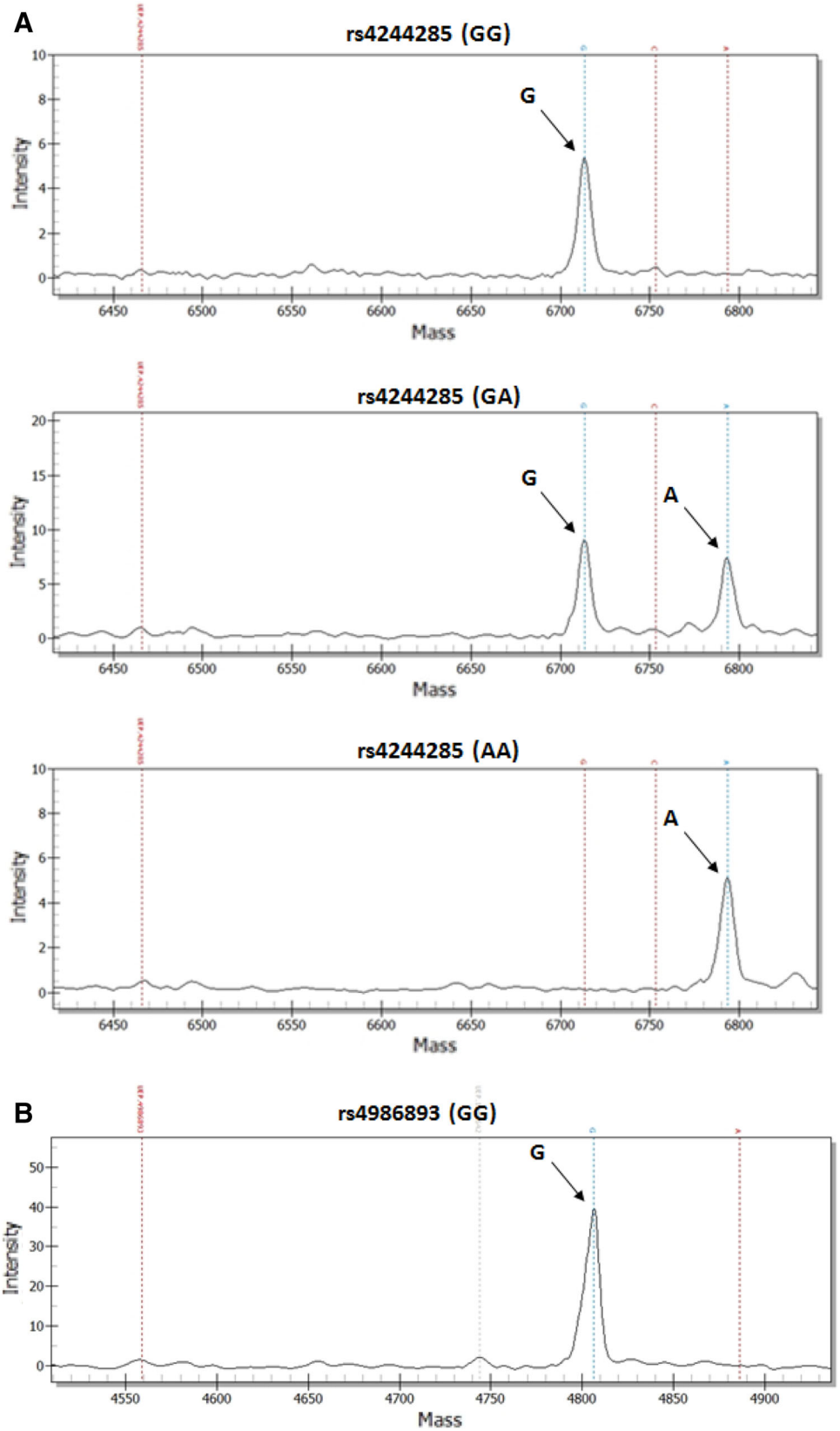


Fig. 1 Mass spectra for genotypes of *CYP2C19* variants rs4244285 ($G > A$), *CYP2C19**2 (**a**), rs4986893 ($G > A$), *CYP2C19**3 (**b**), and rs12248560 ($C > T$), *CYP2C19**17 (**c**); representative examples. For genotype designations, refer to *top* of the *panels*; *peaks* denote particular alleles, *single peaks* represent homozygous, and *double peaks* represent heterozygous combinations

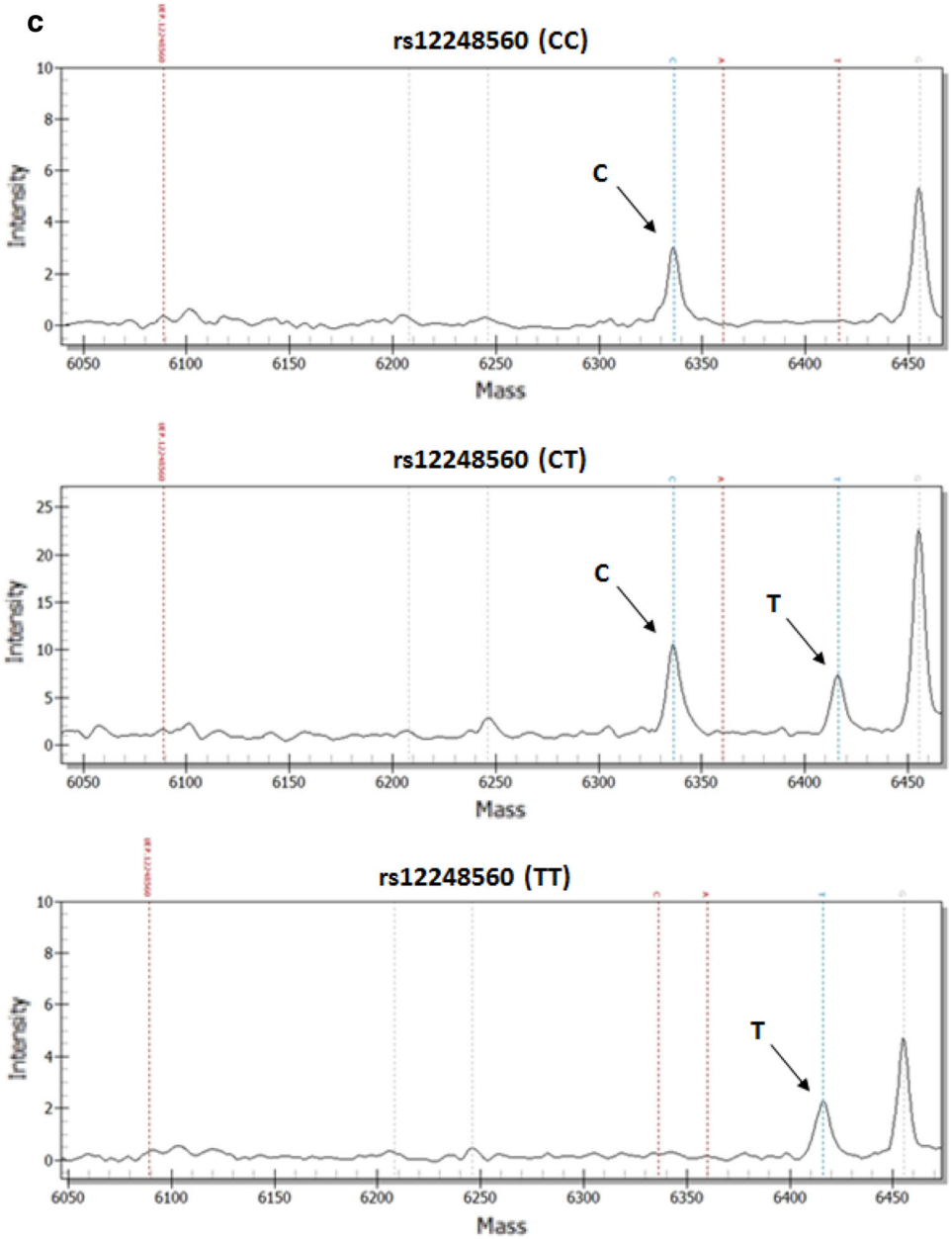


Fig. 1 continued

Statistical Analysis

Each of the three SNPs was tested for Hardy–Weinberg equilibrium (HWE) by Pearson's Chi-square (χ^2) test ($p > 0.05$). Genotype and allele frequencies were determined by direct counting. Carriage rate (phenotype frequency) was calculated as number of individuals carrying one (or two) copies of a particular allele on one or both (maternal and paternal) chromosomes.

Results

CYP2C19 Variant Determination by MassARRAY

All tested *CYP2C19* SNPs could be determined with following success rates: *CYP2C19**2, 99.8%; *3, 99.8%; *17, 99.2%; and, overall, 99.6%. Representative examples of the spectra from MassARRAY are shown in Fig. 1a–c; the distribution of respective genotypes for each of the SNPs is

illustrated by call cluster plots from MassARRAY (Fig. 2a–c). The turn-around-time, from blood sampling to the result print-out, was 12 h in urgent (*statim*) regimen, otherwise from 24 to 36 h.

The distribution of genotypes and alleles of the *CYP2C19* variants in our cohort is summarised in Table 1 (left columns); carriage rates for variant alleles were 0.26 for *CYP2C19**2 A and 0.44 for T allele of *CYP2C19**17. For *CYP2C19**2 and *17 variants, the distributions of genotypes were within HWE ($p > 0.05$); regarding *CYP2C19**3, due to the absence of the variant A allele, χ^2 value could not be defined.

The observed *CYP2C19* variant frequencies in Czechs were fully comparable with those reported for other populations of European descent (Table 1, middle and right columns); the reference population data were obtained from The 1000 Genomes Project (The 1000 Genomes Project Consortium 2015).

CYP2C19 Variant Determination by POC Technique

The success rate for determination of *CYP2C19* variants was 84% (for specific SNPs: *CYP2C19**2: 87%, *3: 83%, *17: 81%). In 10 out of 53 tested samples, the results could not be obtained: there was four times failure during the amplification procedure; three times the report classified results as inconclusive; and in three cases, only two of the three variants could be determined.

Comparison of the genotypes provided by POC with those yielded from parallel examination by MassARRAY showed absolute (100%) concordance. The turn-around-time (from buccal swab sampling to the result print-out) was 60 min.

Relevance for Clopidogrel Metabolism

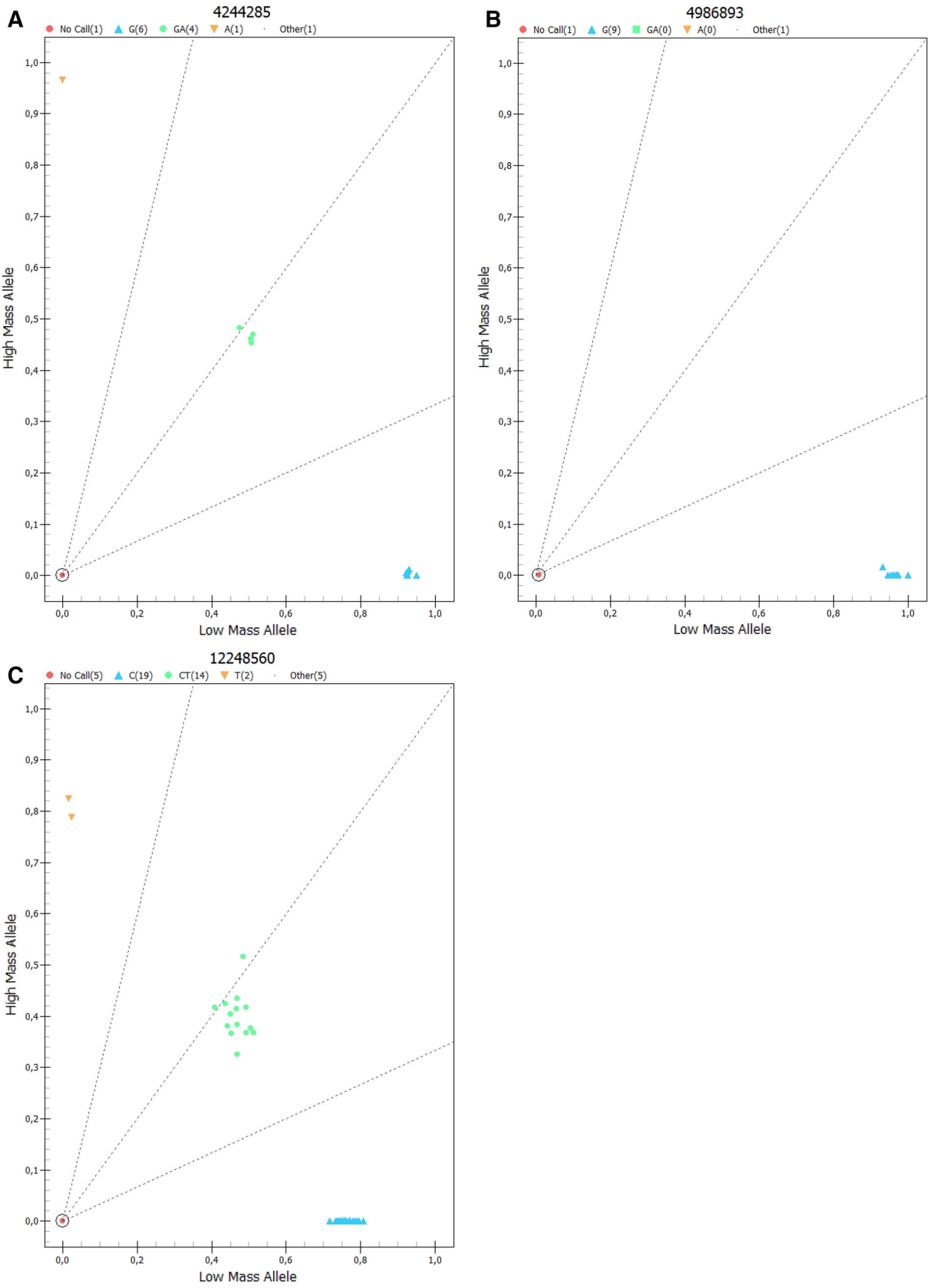
To move from the laboratory genotyping to applied area, the obtained genotypes were divided according to their relevance for clopidogrel metabolism and thus predicted risk of cardiovascular complications (Table 2, left part); the division was based on the classification by Desai et al. (2013). Again, apart from reporting results regarding our study group, we also provide comparisons of the distribution of the particular genotypes in Czechs to other cohorts of distinct ethnicity, more specifically to those of Northern/Southern Europeans and U.S. of European descent (Table 2, middle and right parts). From these comparisons, no substantial differences emerged.

Discussion

Hereby, we present genotyping data on *CYP2C19**2, *3, and *17 variants relevant for clopidogrel metabolism, which were obtained by MassARRAY methodology and in part also by a POC technique in Czech, i.e., Central European cohort counting more than 500 subjects. Both utilised tests proved to be able to determine specific genotypes in a short-time frame (1–12 h) and may, therefore, represent the way towards progress in clinical applications of *CYP2C19* genotyping for management of patients requiring treatment by clopidogrel or alternative platelet inhibitors.

When we compared our Czech data with those reported for other populations of European descent, there were no major differences in the frequencies of individual variants or in the distribution of genotypes relevant for clopidogrel metabolism. In this regard, approximately, a quarter of our patients carried the loss-of-function allele *CYP2C19**2 A, i.e., were poor clopidogrel metabolizers. The A allele is associated with reduced conversion of clopidogrel to active metabolite and these patients are in increased risk of cardiovascular complication including stent thrombosis (Mega et al. 2010b). Another *CYP2C19* loss-of-function allele (*3 A) was not observed in our cohort which was in line with its extremely rare occurrence in Europeans; this allele is present only in Asians with frequency from 1 to 6% (The 1000 Genomes Project Consortium 2015). *CYP2C19**17 TT homozygote individuals were presented in 5%; these "ultra-rapid metabolizers" may have increased risk of bleeding events (Li et al. 2012). Similar to other studies (e.g., Desai et al. 2013), approximately one-third of our patients possessed *CYP2C19* variants phenotypically expressed by alteration of clopidogrel metabolism. Hereby, reported availability of reasonably short-time identification of these variants would, therefore, allow appropriate patient monitoring including consideration of treatment by alternative drugs.

Regarding current initiatives on relocating genotyping from the laboratory to the bedside (Beitelshees 2012; Beitelshees et al. 2015; Knauer et al. 2015), our data may add a small piece into the emerging mosaic. So far, the solid data in area of POC clopidogrel genotyping have been reported by Roberts et al. (2012) whose report suggested that POC testing after PCI can be done effectively at the bedside and be beneficial for treatment of identified CYP2C19*2 carriers with prasugrel; So et al. (2016) recently added report of a POC extended to contain *ABCB1* testing. The presence of Spartan RX and that of another commercial POC test on the market (Nanosphere Verigen System, see http://www.nanosphere.us/products/cyp2c19-test) also provided impetus for development of further


A
4244285
No Call(1) G(6) GA(4) A(1) Other(1)
High Mass Allele
Low Mass Allele
B
4986893
No Call(1) G(9) GA(0) A(0) Other(1)
High Mass Allele
Low Mass Allele
C
12248560
No Call(5) C(19) CT(14) T(2) Other(5)
High Mass Allele
Low Mass Allele

◀**Fig. 2** *Call cluster plots* showing distribution of *CYP2C19* genotypes; representative examples. **a** rs4244285 ($G > A$), *CYP2C19**2; *blue symbols* represent genotype GG; *green symbols* GA; *orange symbols* AA. **b** rs4986893 ($G > A$), *CYP2C19**3; *blue symbols* represent genotype GG, other genotypes not present. **c** rs12248560 ($C > T$), *CYP2C19**17; *blue symbols* represent genotype CC; *green symbols* CT; *orange symbols* TT

alternative POC techniques, such as a microarray-based POC *CYP2C19* genotyping test (Chae et al. 2013) or a recently described chip technique (Marziliano et al. 2015). Before these techniques are readily applied to clinics, there is urgent need for further improvement and standardization including development of relevant external quality assessment schemes as in other fields where genotyping has already been routinely applied to patient care (e.g., Haselmann et al. 2016). In this context, there has been report about specific *CYP2C19* QC outside Europe (Lin et al. 2015).

The strength of our report lies not only within the great number of genotyped subjects, but also regards our main genotyping methodology, which has been controlled by our continuous participation in the external proficiency exercise and the robustness of which we have also documented in other SNP genotyping applications (Kishore et al. 2015, 2016). Regarding limitations of our study, it should be noted that we have involved patients for determination of genotype distribution; however, also our own data confirm that the distribution in patients reflects that in normal reference population (The 1000 Genomes Project Consortium 2015). Otherwise, we did not include other variants known to affect clopidogrel metabolism, such as *ABCB1* (Mega et al. 2010a); however, *ABCB1* gene was not covered by the utilised POC system at the time of our analyses and thus will not enable comparisons.

Importantly, only general knowledge of the Spartan RX POC test methodology is another limiting factor. While with our MassARRAY protocol, we had under

Table 1 Genotype and allele frequencies of *CYP2C19* variants in Czechs in comparison to other European populations

Allele	SNP	Genotype frequencies		Genotype frequencies (EUR)	Genotype frequencies (CEU)	Allele frequencies		Allele frequencies (EUR)	Allele frequencies (CEU)
*CYP2C19*2*	19154 G > A	GG	0.745	0.722	0.758	G	0.864	0.855	0.869
	rs4244285	GA	0.238	0.266	0.222	A	0.136	0.145	0.131
		AA	0.017	0.012	0.020				
*CYP2C19*17*	806 C > T	CC	0.559	0.596	0.586	C	0.753	0.776	0.778
	rs12248560	CT	0.388	0.360	0.384	T	0.247	0.224	0.222
		TT	0.053	0.044	0.030				
*CYP2C19*3*	636 G > A	GG	1.000	1.000	1.000	G	1.000	1.000	1.000
	rs4986893	GA	0.000	0.000	0.000	A	0.000	0.000	0.000
		AA	0.000	0.000	0.000				

EUR European, *CEU* Utah residence with Northern and Western European Ancestry

For the reference population data, see The 1000 Genomes Project Consortium (2015)

Table 2 Distribution of *CYP2C19* genotypes according to their relevance for clopidogrel metabolism phenotypes in Czech and other European cohorts

Clopidogrel metabolism phenotype	CYP2C19 genotype	Population (frequency %)					
		Czech ($n = 526$)	Greek ($n = 283$) (Ragia et al. 2009)	Danish ($n = 276$) (Pedersen et al. 2010)	Faraose ($n = 311$) (Pedersen et al. 2010)	Norwegian ($n = 328$) (Pedersen et al. 2010)	American-USA ($n = 499$) (Desai et al. 2013)
Poor metabolizer	*2/*2	1.7	2.1	2.2	3.2	1.3	3.0
Intermediate metabolizer	*1/*2	23.8	17.7	18.5	23.5	20.1	26.3
Extensive metabolizer	*1/*1, *1/*17	69.2	72.8	67.0	62.4	66.0	65.7
Ultra metabolizer	*17/*17	5.3	3.2	5.1	3.5	4.9	3.2

Classification of metabolism phenotypes according to Desai et al. (2013). For numbers of subjects, see "*n*" next to population designations

control all methodical steps, including reagents' and primer-plexes preparation and handling, with the "closed" nature of the POC system, these and other technical details were unknown, and genotyping was beyond our control from the time-point of inserting the swab into the analyser. Similar situation occurred also in the interpretation phase: while the POC analyser just prints the test outcome, with the MassARRAY, scientists can see the range of parameters including peaks corresponding to amplicons and if relevant they can perform necessary validations. Thus, any explanation of the observed lower success rate of POC genotyping would not be based on solid grounds and will be purely speculative; the same refers to the reasoning for particular statements of the POC system, such as "failure during the amplification procedure" or "inconclusive result" as the QC step of the POC data is not known to us. What we, however, can recommend based on our experience is to carefully respect all steps of the preanalytical phase, especially handling of the swab-stick, performing swab procedure itself, and keeping temperatures and times as described in the SPARTAN RX protocol.

In conclusion, our aggregate data on distribution of main pharmacogenetically relevant *CYP2C19* variants for clopidogrel metabolism obtained by two hereby utilised specific methodologies suggest that there are opportunities for providing genotyping results in a reasonable time frame that can facilitate their application in clinical patient management.

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