Pharmacogenetics of warfarin in a paediatric population: initial dosing and adverse effects (Online Supplementary Data)

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Methods

DNA collection and extraction

Patients provided salivary samples for DNA. DNA was captured, stabilised and purified using the Oragene.DNA kit [42]. Following collection of the sample, and mixing with the Oragene DNA preserving solution, samples were stored at -80°C. Following defrosting, samples were incubated (50°C, 1 hour), and then Oragene DNA Purifier added (1:25), followed by incubation (ice, 10 minutes). The mixture then was centrifuged (4600rpm, 10 minutes). The supernatant resulting was mixed with an equal volume of 95% ethanol, ceintrifuged (4600rpm, 10 minutes). The resulting DNA pellet was rinsed with 1ml 70% ethanol, and rehydrated (addition of TE buffer, cold room storage). Assessment of rehydration was made at 7 days, if incomplete, additional cold storage (7 days) undertaken. Quantification was undertaken using Nanodrop.

Genotyping

The SNPs CYP2C9*2 (rs 1799853), CYP2C9*3 (rs 1057910) and VKORC1 (rs 9923231) were selected for genotyping, as these have been shown to affect dosing requirement in adult populations using warfarin [8-10].

The genotyping of CYP2C9*2, CYP2C9*3 and rs9923231 was performed on ABI 7900HT Real time PCR using Taqman chemistry. PCR was carried out using Taqman Drug metabolism Genotyping Assays C-30403261_20, C-25625805_10 and C-30403261_20. A reaction volume of 10µl contained 10ng DNA, 1xTaqman master mix and 1 x Taqman drug metabolising genotyping assay mix. In order to minimise cross contamination of samples, a dry-down DNA method was performed according to manufacturer's protocol. The PCR condition were as follows: Activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 15 sec and extension at 60°C for 90 sec. Alleles were clustered using fluorescent signals (VIC and FAM). Each PCR plate was contained 10% duplicates, two negative controls and one heterozygous control for each SNP and samples with a discrepant call were repeated under the same conditions. Self-priming of negative control(s) was considered to be contamination, and genotyping repeated. Genotype personnel were blinded to outcome data.

Supplementary table 1

Target (range) of international normalized ratio (INR)	Clinical Indication
2.0 (1.5-2.5)	Haemodialysis
2.5 (2.0-3.0)	Fontan's Circulation, Cavopulmonary anastomosis, Central Venous line thrombosis, Pulmonary embolus, Proximal DVT, Calf Vein Thrombosis, Recurrence of Venous Thromboembolism, Non-rheumatic Atrial Fibrillation, Mural Thrombus, Cardiomyopathy, Cardioversion, Symptomatic Inherited Thrombophilia
3.5 (3.0-4.0)	Recurrence of venous thromboembolism whilst on Warfarin therapy, Mechanical Prosthetic Valve, Unfenestrated Fontan Circulation

Targets and Ranges of INR for various clinical conditions used at Alder Hey Children's Hospital (adapted from "Guidelines for oral anticoagulation" [4])

Supplementary data table 2

	N	Mean	Std. Deviation
Age	12	7.2	7.1
Height (cm)	11	1.2	0.412
Weight (kg)	12	30.1	29.6
Albumin (g/dl)	10	33.7	4.8
BMI (kg/m^2)	11	18.9	5.7
Male gender	7/12	-	-

Characteristics of the 12 patients who did not achieve stable dose (at the start of therapy).

Supplementary table 3

	VKORC1Wild Type	VKORC1 -1639 Heterozygote	VKORC1 -1639 Homozygote
CYP2C9*1/*1	23	32	6
CYP2C9*1/*2	5	6	5
CYP2C9*2/*2	2	0	0
CYP2C9*1/*3	9	5	1
CYP2C9*2/*3	1	2	0

The combination of genotypes found in the cohort of children recruited

Supplementary Table 4

Individual SNP association analyses

Outcome	SNP (assumption)	p-value from LRT	Significant following FDR adjustment
Proportion of time in INR range (PTIR)*	*CYP2C9*2* (none)	0.58,0.030	No
	*CYP2C9*2* (additive)	0.53	No
	*CYP2C9*3* (NA†)	0.95	No
	VKORC1-1693 (none)	0.03, 0.014	No
	VKORC1-1693 (additive)	**0.001**	**Yes**
INR above range in week 1	*CYP2C9*2* (none)	0.040,0.988	No
	*CYP2C9*2* (additive)	**0.004,**	**Yes**
	*CYP2C9*3* (NA†)	0.800	No
	VKORC1-1693 (none)	0.23, 0.031	No
	VKORC1-1693 (additive)	0.020	No
Stable dose**	*CYP2C9*2* (none)	0.089, 0.802	No
	*CYP2C9*2* (additive)	**0.008**	**Yes**
	*CYP2C9*3* (NA†)	0.049	No
	VKORC1-1693 (none)	0.35, 0.03	No
	VKORC1-1693 (additive)	**0.003**	**Yes**
Haemorrhagic complications	*CYP2C9*2* (none)	0.819, 0.989	No
	*CYP2C9*2* (additive)	0.423	No
	*CYP2C9*3* (NA†)	0.482	No
	*VKORC1-1693*2* (none)	**0.006, 0.87**	**Yes**
	VKORC1-1693 (Additive)	0.288	No

Individual SNP association analyses. FDR = false discovery rate; LRT = likelihood ratio test.

* Analyses adjusted for indication for treatment and target INR group

** Analyses adjusted for age and target INR group

† No mutant homozygotes so assumption regarding mode of inheritance irrelevant

Supplementary Data Table 5

Outcome	Assumed mode of inheritance	Predictor variables	Coefficient (95% CI)
Proportion of time in INR range	None	CYP2C9*2 (1)	0.02 (-0.12, 0.15)
		(2)	-0.36 (-0.74, 0.02)
		VKORC1*2 (1)	0.11 (-0.00, 0.23)
		(2)	**0.20 (0.02, 0.39)***
		Indication for treatment	0.09 (-0.03, 0.20)
	Additive	VKORC1*2	**0.11 (0.04, 0.19)***
		Indication for treatment	0.11 (-0.01, 0.22)
Stable dose	None	VKORC1*2 (1)	-0.44 (-1.30, 0.43)
		(2)	**-1.33 (-2.55, -0.11)***
		Height	**2.10 (0.38, 3.82)***
		Target INR (1.5-2.5)	0.76 (-0.71, 2.22)
		(2.0-3.0)	0.56 (-0.93, 2.05)
		(3.0-4.0)	-0.91 (-3.28, 1.47)
		Indication for treatment (1)	-0.25 (-1.50, 1.00)
		(2)	-0.61 (-2.19, 0.96)
	Additive	VKORC1*2	**-0.60 (-1.17, -0.03)***
		Height	**2.08 (0.37, 3.78)***
		Target INR (1.5-2.5)	0.77 (-0.70, 2.23)
		(2.0-3.0)	0.51 (-0.96, 1.99)
		(3.0-4.0)	-0.93 (-3.30, 1.44)
		Indication for treatment (1)	-0.25 (-1.50, 0.99)
		(2)	-0.64 (-2.21, 0.92)

Multiple SNP regression models (continuous variables) *$p < 0.05$

(1) Heterozygote for allele (2) Homozygote for allele

Supplementary Data Table 6

Outcome	Assumed mode of inheritance	Predictor variables	Odds ratio (95% CI)
INR above range in week 1	None	CYP2C9*2 (1)	2.83 (0.91, 8.85)
		(2)	-
		VKORC1*2 (1)	2.12 (0.82, 5.49)
		(2)	**7.25 (1.25, 41.98)***
	Additive	CYP2C9*2	**3.59 (1.32, 9.78)***
		VKORC1*2	**2.38 (1.16, 4.92)***
Haemorrhagic complications	None	VKORC1*2 (1)	**3.50 (1.31, 9.32)***
		(2)	0.88 (0.16, 4.90)
	Additive	NA	NA

Multiple SNP logistic regression models (binary variables) *$p < 0.05$. (1) Heterozygote for allele (2) Homozygote for allele

Supplementary Table 7

Outcome	Variables included	Coefficient (95% CI)*	Adjusted/pseudo R^2		
			Non-genetic variables	Genetic variables	All variables
PTIR	Indication for treatment	0.09 (-0.04, 0.22)	11.3%	9.5%	20.8%
	INR group	1: -0.33 (-0.54, -0.12)			
		2: -0.00 (-0.17, 0.17)			
		3: 0.20 (-0.08, 0.49)			
	VKORC1-1693 (additive)	0.13 (0.05, 0.21)			
INR exceeding target range in week 1	*CYP2C9*2* (additive)	4.18 (1.42, 12.34)	-	6.8%	6.8%
Stable dose	Age	0.19 (0.12, 0.25)	29.2%	11.9%	41.4%
	INR group	1: -1.05 (-2.36, 0.26)			
		2: 0.93 (0.14, 1.73)			
		3: -0.27 (-2.81, 2.27)			
	*CYP2C9*2* (additive)	-0.82 (-1.39, -0.25)			
	VKORC1-1693 (additive)	-0.66 (-1.08, -0.25)			
Haemorrhagic complications	*VKORC1-1693* (none)	Hetero: 4.53 (1.59, 12.93)	-	8.7%	8.7%
		Homo: 1.13 (0.20, 6.51)			

Final multiple regression models
*Regression coefficient for multiple regression models; odds ratio for logistic regression models;
CI = confidence interval. PTIR = Proportion of time spent in target INR range.