CHAPTER VII

SUMMARY AND DISCUSSION

7.1 THIS THESIS

When this project commenced, comprehensive whole genome genotyping chips were not yet available, the HapMap project was just scaling up, and genotyping was much more expensive than it is now thus, the candidate gene approach was therefore a realistic route to search for the genetic determinants which influence warfarin dose and the cause of ADR of bleeding. Throughout the project, a total of 35 candidate genes were selected based on pharmacokinetics and pharmacodynamics of warfarin reported in the literature (Chapter III). In the initial phase of SNP selection, only a small number of SNPs in public databases had allele frequency information attached to them. LD maps were constructed for each gene or locus (e.g. CYP2C genes on chromosome 10) using a sample of 201 warfarin treated patients enrolled by the University of Uppsala in Sweden. The maps harbour 379 common SNPs (MAF≥5%) and capture most of the common variation in HapMap ($r^2 \geq 0.8$). SNP genotyping was carried out using the MassExtend and iPLEX assays from Sequenom.

Our group's collaboration with Drs. Mia Wadelius (Uppsala) and Anders Rane (Karolinska) gave us access to two study samples:

- 201 warfarin treated patients (including 12 bleeding patients) plus 24 additional patients with bleeding complication (Uppsala study)

- 1496 warfarin treated patients including 28 bleeders enrolled in the prospective WARG study in Sweden.

In both studies patient information was available on age, gender, indication, concomitant medication, and corresponding dose and PT INR values in each visit to clinics.

We first looked for genetic determinants of warfarin dose requirements (Chapters IV and V). After two rounds of discovery / replication in the Uppsala and WARG sample collections, only two of the 35 candidate genes interrogated showed irrefutable evidence for a strong effect on inter-individual dose variation. Both these genes namely *VKORC1* and *CYP2C9* encode peptides that directly interact with warfarin. Others and we have demonstrated that the *VKORC1* genotype and *CYP2C9* *2 / *3 alleles have predictive value justifying their usefulness as a genotyping test for patients who are prescribed warfarin. Based on the evidence we provided of *VKORC1* rs9923231 and *CYP2C9*2 and *3 association with (1) stable dose, (2) time to reach stable dose, and (3) risk of over-anticoagulation, their use in a clinical setting will narrow the time window for reaching an optimal warfarin dose and reduce hospitalisation. The genetic impact of the two genes is detailed in the respective chapters (chapters IV and V).

In the Uppsala study we also observed associations with warfarin dose for *APOE*, *EPHX1*,

CALU, *PDIA2*, *GGCX*, *ORM1-2* and *PROC*; the latter almost reached experiment-wise significance. However, upon replication in the prospective WARG study of 1496 patients also of Swedish origin, none of these genes reached significance after correction for multiple tests (see Table 5.5). Minor effects from *GGCX*, *EPHX1*, *APOE* and *ORM1-2* have shown a replication trend in both this and other studies (Kohnke et al. 2005; Rieder et al. 2007; Sconce et al. 2006; Wadelius et al. 2007). Our data suggest that these genes do not have a strong effect on dose and are potentially population/treatment specific.

Based on genetic factors determined through the study and in combination with non-genetic factors such age, gender and drug interaction, nearly 60% in dose variation could be explained. However the remaining 40% still needs to be deciphered. It might be due to both genetic factors in other untested genes and environmental factors such as diet for which our studies had not recorded sufficient information. Some relevant non-genetic factors could be recorded using proteomic technologies such as measuring peptide and chemical compositions in the serum and urine.

The ultimate goal of this project is to develop a warfarin-dosing algorithm. It is relevant to mention the work of Niclas Eriksson (Uppsala), which has led to the development of such a tool based on our knowledge. The calculated coefficients of each predictor in his dosing

algorithm are listed in Table 7.1. The number calculated according to this table needs to be squared, which is the predicted weekly maintenance dose.

Table 7.1. Predictors for warfarin dosing algorithm.

Coefficients		Estimate	Standard error	P value
Starting		9.46832	0.11867	$<2x10^{-16}$
CYP2C9	*1/*2	-0.50836	0.05811	$<2x10^{-16}$
	*1/*3	-0.97546	0.07077	$<2x10^{-16}$
	*2/*2	-1.10204	0.19767	$3.0x10^{-8}$
	*2/*3	-1.74761	0.20391	$<2x10^{-16}$
	*3/*3	-3.40061	0.33091	$<2x10^{-16}$
VKORC1 rs9923231	A/G	-0.90112	0.04959	$<2x10^{-16}$
	A/A	-2.01863	0.06799	$<2x10^{-16}$
Age at start		-0.03686	0.00172	$<2x10^{-16}$
Female gender[1]		-0.27698	0.04682	$4.2x10^{-9}$
Interaction[2]		-0.06992	0.01867	0.00019

For example, a 60 year old man with no interacting drugs is genotyped as *CYP2C9* *1/*3 and *VKORC1* rs9923231 A/A alleles. The calculation is 9.46832 + (-0.97546) + (-2.01863) + (-0.03686)*60 + (-0.27698)*0 + (-0.06992)*0 = 4.26263. By squaring the obtained number, the predicted dose for this 60 year old man is 18.17 mg/week. Hopefully, new findings will improve the predictive power of such tools.

In chapter VI, the analysis of the severe bleeding complication phenotype which occurs in

~2% of warfarin treated patients as a result of using this drug is presented. The results suggest that bleeding is likely to be a complex trait, but this needs to be further addressed. Due to the small size of bleeding patients (64 in total) our analyses lacked statistical power, and further samples will be needed to replicate some of the initial findings. Three members of the protein disulfide isomerise gene family, *P4HB*, *PDIA4* and *PDIA6*, may be implicated in warfarin-induced severe bleeding complication and will be of interest to pursue further including some functional characterisation.

For reasons outlined in Chapter VI, 11 of the genes (see Table 6.20) were selected for exon re-sequencing in 48 warfarin treated patients including 36 bleeders and 12 non-bleeders, as well as 48 CEPH Caucasians. To complete our knowledge of common variants possibly enriched in bleeders i.e. no high penetrance variants which cause bleeding complication, investigation of rare functional variants was also performed. Compared to typical Mendelian disorders, the 2% prevalence of bleeding among warfarin treated patients is relatively common and it is more likely to be caused by a common, possibly low frequency (1-5%) variant. Due to the small number of samples sequenced, the common SNPs we identified in the Swedish patients but not in the 48 CEPH individuals may be of interest and need to be further assessed in all patients in the WARG study.

The findings from studying 35 candidate genes were described in this thesis. With the latest developments in genotyping technology, genome-wide association (GWA) studies have become both feasible and cost effective in large sample sizes (Amos 2007). The WARG study has a reasonably large sample size to provide statistical power to detect moderate effects in a genome-wide scan for warfarin dose. Our lab and the collaborating groups in Sweden have come to the view that a GWA approach is the way forward. The first 1000 samples of the WARG study have been scanned with the Illumina Hap370K array. Initial analysis indicated that there are no loci with effects comparable to *VKORC1* and *CYP2C9* (N Soranzo and P Deloukas, personal communication). This is in agreement with a report by Rieder and colleagues presented in the recent Pharmacogenomics meeting (2007) held in Hinxton, United Kingdom, in which an underpowered genome scan of 186 samples with the Illumina 550K chip did not yield any statistically significant findings. The way forward will be to combine many more genome scans and to increase power to detect small effect sizes.

7.2 MOLECULAR MECHANISM

Studies aiming to link genotype to phenotype often bypass many intermediate steps which are biologically relevant. The study of gene expression as intermediate phenotype is well documented in the recent literature (Nevins and Potti 2007; Ozdemir et al. 2006). Expression

data can directly point to new candidate genes based on their response to a stimulus under investigation, e.g. a drug such as warfarin. This information can become even more powerful once combined with genome-wide data on sequence variants (Goring et al. 2007; Stranger et al. 2007).

I have initiated work with two well-established human hepatoma cell lines, Hep G2 and Hep 3B, which I treated with isomeric mixtures of warfarin. The warfarin-treated cell lines could provide systematic information as to which genes are up- or down-regulated in response to warfarin *in vitro*. A previous study has demonstrated the accumulation of des-r-carboxyprothrombin or proteins induced by vitamin K antagonism (PIVKA-II) in Hep G2 cell hepatoma in the presence of warfarin (Lawley et al. 2006). It is planned to look at the effect of warfarin treatment on gene expression in hepatoma cells as previously described by Lawley et al.

This will help understand the molecular interaction when warfarin is administered in patients. Genes showing differential expression response could be influential to, or as an outcome of, inter-individual dose variation and bleeding ADR. We anticipate that such data can be used to provide additional statistical weight to weakly associated SNPs in the genome scan. Although the experiment is done with human hepatoma, this experiment would be best done using

primary hepatocytes as hepatocytes retain a more complete drug metabolising system.

7.3 CONCLUDING REMARKS

In August 2007, the US FDA updated the label of warfarin to include information on pharmacogenetic testing. The encouragement, but not compulsoriness, of genetic testing before initiating warfarin therapy is one of the most debated subjects. This thesis provided results that have contributed to the body of evidence used by the US FDA to reach this decision and in a broader sense to pharmacogenomic research towards personalised medicine.