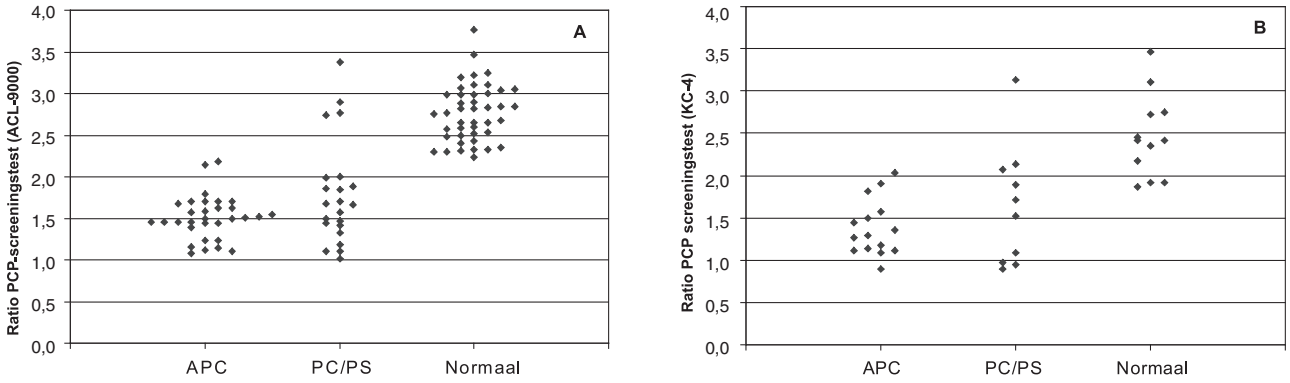

Figuur 1. Weergave van de ratio's van de PCP screeningstest voor de 3 patiëntencategorieën. APC; patiëntenmonsters met een aangetoonde F-V-Leiden mutatie, PC/PS; patiëntenmonsters met een verlaagd PC/PS, Normaal; patiëntenmonsters waarbij trombofiliescreening is aangevraagd en geen afwijkingen gevonden zijn.

niveau van 61% niet als afwijkend geduid. Het is echter de vraag wat de klinische relevantie van zulke PS-niveau's is en of het detecteren van een afwijkend PS, gedefinieerd als < 50%, als controle meegenomen zou dienen te worden om hiermee een uitgevoerde run van patiëntenmonsters goed dan wel af te keuren. Binnen het Rijnland Ziekenhuis is ervoor gekozen om daarom bij elke run een PC- en PS-deficiënt plasma 1:1 verdund met normaalpool mee te nemen, waarbij de PC- en PS-niveau's tussen de 50 - 55% liggen.

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Detection of the 1173C>T polymorphism of the human Vìtamin K epoxide reductase complex (VKORC1) gene by LightCycler real-time PCR

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Recently it has been demonstrated that polymorphism's in the vitamin K epoxide reductase complex subunit 1 gene (VKORC1) is associated with interindividual differences in the response to coumarin anticoagulants (1-4). The human VKORC1 gene encodes a 163 amino-acid enzyme located in the endoplasmic reticulum (GenBank, AY587020) (4). A recent study demonstrated that genetic polymorphism's in VKORC1 are responsible for about 37% of the variability in the anticoagulant pharmacological response. The VKORC1 1173C>T polymorphism accounts for a low dose requirement and therefore genotyping for VKORC1 can predict a high risk for overdose before initiation of anticoagulation (2).

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The aim of this study was to develop a real-time PCR followed by melting curve analysis, using hybridization probes with a highly sensitive, rapid and efficient approach to mutation detection. The LightCycler instrument (LC) was used for the detection of the single nucleotide polymorphism 1173C>T of the human VKORC1 gene. To evaluate the reliability of genotyping with the LightCycler the samples were also analyzed for the VKORC1 1173C>T polymorphism by digestion with a restriction enzyme.

Material and methods

DNA was extracted from 400 µl EDTA blood and eluted in 200 µl elution buffer according to the manufacturer's protocol with a MagNaPure Compact (Roche Diagnostics). Anonymous DNA samples with a known VKORC1 genotype were received from an external laboratory. For the detection of the 1173C>T polymorphism, PCR primers amplified a 138 bp fragment of the VKORC1 gene. During PCR the amplicon was detected using two specific hybridization probes,

Table 1. Oligonucleotides (5'-3') used for the detection of the 1173C>T polymorphism in the VKORC1 gene

	position	length	GC (%)
primers			
ggATAgggTCAgTgACATggAAT	6419-6441	23	48
gCCCgAgAAAggTgATTT	6556-6539	18	50
probes			
CCATCCTAgTCCAAgAgTCgATgATCTCC--FL	6499-6471	29	51.7
LC Red640-ggCACCgggCACCTTTggCC--PH	6469-6450	20	75.0

one labeled with fluorescein and one with LightCycler Red 640 (LCRed640). The presence of a C-allele introduces a destabilizing mismatch, which results in a decreased melting temperature. Primers and probes were designed and custom-made by TIB MOLBIOL, Berlin, Germany. The sequences are given in Table 1. For mutation detection with the LightCycler, a 20 µl reaction was performed. The reaction mixture contained 1x LightCycler DNA Master Hybridization Mix, 2.5 mM $MgCl_2$, 10 pmol of each primer, 8 pmol of each hybridization probe, and 2µl genomic DNA (125 ng).

PCR cycling conditions were: 120 seconds at 95 °C for DNA denaturation, 50 cycles (5 seconds at 95 °C (denaturation), 8 seconds at 55 °C (annealing), 10 seconds at 72 °C (extension)). After the PCR, a melting curve analysis was performed by heating to 95 °C for 20 seconds, followed by cooling to 40 °C for 20 seconds and gradual heating to 85 °C at 0.2 °C/s. After the melting curve analysis a final cooling was performed at 40 °C for 30 seconds. To analyze the melting curves the corresponding melting peaks were calculated by plotting the first negative derivative of the fluorescence with respect to the temperature (-dF/dT vs. T).

For restriction enzyme digestion (*Hinf* I, Roche Molecular Biochemicals) DNA was amplified by conventional PCR in a T3 thermocycler (Biometra). The 50 µl PCR reaction mixture contained: 1x PCR buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 10 pmol of each primer, 1U Taq polymerase (Perkin Elmer) and 5 µl genomic DNA (250 ng). The PCR cycling conditions were: 5 minutes at 95 °C, followed by 35 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds. The PCR products were digested for 2 hours at 37 °C in a total volume of 15 µl containing 1x SuRE/Cut buffer H, 5 U *Hinf* I (Roche) and 10 µl PCR product. After digestion the fragments were separated on a polyacrylamide gel (PAGE) and visualized with silver staining.

Results

A LightCycler assay was developed to analyze the VKORC1 1173C>T polymorphism. The PCR products were tested on an agarose gel and the amplicons showed the expected size (138 bp). During the melting curve analysis the probes were dissociated from the target DNA. In case of a T-allele (mutant genotype) the fluorescein-labeled probe has a fully homologous sequence with the target DNA and was dissociated from the target DNA at a melting temperature of 70.5 °C. In case of a C-allele (wild-type genotype) one mismatch occurs and the melting temperature was decreased to 66.2 °C (Fig. 1). Within 10 different experiments the variation in melting temperature was on average less than 1 °C. In heterozygous genotypes the difference in melting temperature (Δ between melting peaks) was 4.4 °C ± 0.75 °C (46 samples).

The restriction enzyme *Hinf* I recognizes the sequence 5'G ↓ ANTC3' and in case of a T-allele an extra recognition site is created.

In case of a C-allele the PCR-product is digested in two bands: 118 bp and 20 bp. In case of a T-allele the PCR-product is digested in three bands: 75 bp, 43 bp and 20 bp. Heterozygous samples show four bands: 118 bp, 75 bp, 43 bp and 20 bp. On the polyacrylamide gel the 20 bp band could not be detected but the difference between an undigested PCR-product and a digested C-allele was obvious.

To evaluate the reliability of genotyping with the LightCycler 25 human DNA samples (including three samples with a known genotype) were analyzed for VKORC1 1173C>T polymorphism with the LightCycler and by digestion with *Hinf* I. Genotyping 25 DNA human samples resulted in 9 CC, 11 CT and 5 TT alleles. With both methods in all samples tested a clear genotype was obtained and no discrepancies between the two methods were found.

Discussion and conclusion

With the described LC assay it is possible to detect the VKORC1 1173C>T polymorphism. Comparison between LightCycler genotyping and digestion with a restriction enzyme showed complete concordant

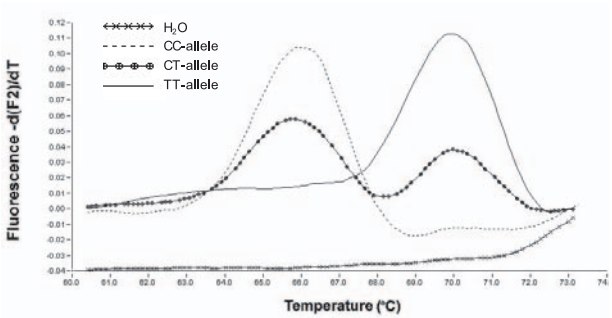

Figure 1. Melting peaks for VKORC1 1173C>T polymorphism. Homozygous wildtype (CC-allele) T_m at 66.2 °C, homozygous mutant (TT-allele) T_m at 70.5 °C, heterozygous sample (CT-allele) T_m at 66.2 °C and 70.5 °C.

results. Performing a LightCycler assay in a closed system eliminates post-amplification processing. This considerable reduction in the number of manipulations reduces the risk of contamination. The use of the LightCycler clearly reduced analysis time (45 minutes vs. 1.5 days).
In conclusion real-time PCR followed by melting curve analysis is a rapid, simple, accurate method for genotyping the VKORC1 1173C>T polymorphism.

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Automated result interpretation in anemia testing using artificial neural networks

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Artificial neural networks (ANNs) are non-linear statistical data processing tools based on the simulation of groups of interconnected neurons, which work analogously to biological neural nets. An ANN can be trained for a particular task by repetitively adjusting inter-neuronal connection weights so that the discrepancies between output and true values are minimized. ANNs can be applied in complicated classification tasks, and show promise for application in medical decision making (1,2). In our laboratory, anemia test results ordered by general practitioners are reported with classification codes referring to the most probable cause of anemia. The coding is done non-automated by a clinical chemist, is time intensive and probably operator dependent. In this study, we examined the abilities of two ANNs software packages to learn this particular task, in order to explore the feasibility of automated interpretation of laboratory results.

Methods

Two software programs were used, both being implementations of a standard feed-forward back-propagation ANN model. Nets were created with one input layer containing a number of neurons equaling the number of input parameters, two hidden layers with variable numbers of neurons, and one output neuron signaling the likelihood of a particular classification. Dedicated ANNs were employed in parallel for the classification codes: iron deficiency, thalassemia, infection, blood loss, pregnancy, decreased erythropoiesis, and uncertain/unknown cause. The general principle is shown in figure 1. Separate datasets were used for training, for validation (detecting potential overtraining) and for evaluation, and only contained test results ordered by general practitioners. Data were obtained from the LIS and a Sysmex XE-2100 hematology analyser.
The first program tested was NNclass, a freeware MS-Excel implementation (3). Nets were defined with randomly sized hidden layers. Input parameters included: age, sex, ESR, zinc protoporphyrin (ZPP), Hb, RBC, MCV, MCH, RDW-SD, neutrophilic granulocytes, immature reticulocytes fraction and reticulocytes. Missing input data were substituted by mean values obtained from the training dataset. Training was performed for 2000 cycles using 649 training and 115 validating examples. ANNs with the lowest training- and validation error rates were selected, feeded with an evaluation dataset (n=170), and evaluated for the subjective acceptability of their output. The analytical performance was evaluated with a separate dataset (n=431).
EasyNN-plus (4), a commercial stand-alone application, was also tested. Hidden layer size was optimized by the program prior to learning. Input parameters were: age, sex, ESR, ZPP, Hb, RBC, MCV, RDW-SD, platelets, granulocytes, lymphocytes, monocytes, IRF and reticulocytes. Missing input data were substituted by median values

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