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Genotype-based screening for hereditary haemochromatosis. I: Technical performance, costs and clinical relevance of a German pilot study

Manfred Stuhrmann*,[1], Christian Strassburg[2] and Jörg Schmidtke[1]

[1]Institute of Human Genetics, Hannover Medical School, Hannover, Germany; [2]Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

In 2001, we initiated a pilot study on DNA-based screening of hereditary haemochromatosis (HH) in Germany. A total of 5882 insurants of the German sickness fund Kaufmännische Krankenkasse–KKH requested information on this project, and 3961 of these individuals provided blood samples for testing of the HFE mutation C282Y. Of these, 3930 samples were successfully tested with two independent test methods, and the results were communicated to the referring doctors. In all, 67 of the tested individuals were homozygous for C282Y. Partially, this high rate (1.7%) can be explained by the fact that 42.6% of the homozygotes already knew their clinical diagnosis HH before sending the blood sample. Iron accumulation with further signs or symptoms of HH was present in eight of 34 newly diagnosed C282Y homozygous individuals. Two major aspects of our study were the analytic validity and the direct laboratory costs of different test methods. Of 7860 tests performed, 7841 (99.6%) gave correct results. The overall error rate was 0.24% (95% CI: 0.15–0.38%). The analytic specificity of the tests methods with respect to the detection of homozygosity for C282Y was 100% (7726 of 7726 nonhomozygous test challenges, 95% CI: 99.95–100%), while the analytic sensitivity was 97% (130 of 134 homozygous test challenges, 95% CI: 92.5–99.2%). The direct costs ranged from 11.20–16.35 € per test method. We conclude that the test methods for C282Y are robust, highly sensitive and specific, and that a DNA-based HH-screening program can be performed at reasonable laboratory costs.

Introduction

Hereditary haemochromatosis (HH, [MIM 235200]) is characterised by an overabsorption of iron from the small intestine tract and the progressive accumulation of iron in most body tissues, resulting in cellular and organ damage, in particular of the liver. Early symptoms include variable degrees of fatigue, impotence, arthralgias or arthritis, and in later stages an elevation of serum level of liver enzymes.[1,2] Later, patients may experience a bronze skin pigmentation, arthropathy, cardiomyopathy, endocrine disorders (mainly diabetes mellitus and hypogonadism), and liver cirrhosis as well as hepatocellular carcinoma, which leads to a severe reduction in life expectancy. Early diagnosis is therefore desirable, since iron removal by phlebotomy is highly effective, improving survival as well as reducing morbidity, and, when started before the development of cirrhosis or diabetes, is likely to lead to a normal life expectancy.[2]

In 1996, a candidate gene for HH was identified near the major histocompatibility complex (MHC) on chromosome

*Correspondence: Professor M Stuhrmann, Institute of Human Genetics, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany. Tel: +49 511 532 3719; Fax: +49 511 532 5865;
E-mail: stuhrmann.manfred@mh-hannover.de

6p.[3] This gene was initially termed *HLA-H* and later renamed *HFE*. Depending on the population under study, between 64 and 100% of HH patients (reviewed in Burke *et al*[4]) were found to be homozygous for a G to A transition at nucleotide 845 of the *HFE* gene resulting in a cysteine to tyrosine substitution at amino acid 282 (C282Y).[3] C282Y represents the major HH mutation, but two other lowly penetrant alleles (H63D[3], S65C[5]), and several rare mutations have meanwhile been identified in the *HFE* gene.

The frequency of HH homozygosity in Caucasians is estimated to be 2–5 per 1000.[6-8] Because of the high frequency of HH, the availability of simple test methods and an effective treatment, population screening has been advocated.[7,9-11] In 1997, a panel with expertise in epidemiology, genetics, hepatology, iron overload disorders, molecular biology, public health and the ethical, legal, and social implications of genetic information was convened by the Centers for Disease Control and Prevention (CDC) and the National Human Genome Research Institute (NHGRI). Its task was to review evidence regarding the clinical presentation, natural history, and genetics of HH and on the ethical and health policy implications of DNA-based testing for HH.[4] This panel came to the conclusion that genetic testing is not recommended at this time in population-based screening for HH due to uncertainties about prevalence and penetrance of HFE mutations, and the optimal care of asymptomatic people carrying HFE mutations. However, the panel accorded high priority to population-based research to study the genotype–phenotype correlation in HH and to investigate the ethical, social and psychological effects of DNA-based testing.[4]

In 2001, we initiated a pilot study to investigate the validity and costs of different genetic test methods, as well as the clinical relevance, acceptance, uptake rates of consultations, counselling and testing, and the ethical and social consequences of DNA-based HH screening in Germany. Here, we focus on the costs, the technical and clinical aspects of the project. The other aspects of our pilot study will be published subsequently.

Materials and methods
Study design and participants
The study was performed in conjunction with a German sickness fund, the health insurance company Kaufmännische Krankenkasse–KKH, which is the nationwide insurer of 1. 4 million adult individuals (63% female, 37% male). Brief information on the pilot project, the clinical picture of haemochromatosis and the availability of genetic testing and treatment was given to the insurants via the magazine 'KKH-Journal' in all of four issues of the year 2001, as well as by displaying flyers in the KKH offices. The KKH journal contains articles on the company's activities and general health care and is sent free of charge

to every insurant of the KKH. Insurants who read the article on the pilot project and who wished to obtain more information were invited to contact the Institute of Human Genetics at Hannover Medical School (MHH) by telephone, Fax or mail. All individuals who expressed interest received an information leaflet, together with a consent form, a filter paper and the offer to attend to the monthly information meetings at MHH and to receive pretest counselling (personal or via telephone). Individuals who considered taking part in the pilot project were invited to discuss this issue with their physicians, usual general practitioners (GP), at the next regular visit. From those participants who gave written informed consent, capillary blood was taken by the GP, spotted on the filter and sent to the MHH. After completion of the test, the result was sent to the GP, together with (i) the offer for genetic counselling in all cases of homozygosity or heterozygosity and (ii) a questionnaire on symptoms as well as clinical and laboratory findings in all cases of homozygosity. Neither the names of the participants nor individual results were given to the KKH.

The study was approved by the institutional review board (IRB) of the MHH and by the 'Bundesversicherungsamt (BVA)', the independent federal commission exercising oversight over the nationwide German sickness funded system. An external independent advisory board, consisting of Professor Bayertz (Ethics, University of Münster), Professor Stremmel (Internal Medicine, University Hospital Heidelberg) and Professor Vogel (Human Genetics, University of Ulm), supervised the project.

Laboratory analysis
Capillary blood was spotted on filter paper (ISOCODE® cards; Schleicher & Schuell, Dassel, Germany) and sent to the MHH by regular mail. Isolation of DNA was performed according to the manufacturers' protocol.

Each DNA sample was tested for presence of the mutation C282Y with two of the following test methods. Although principally possible with some of the following methods, we did not evaluate the presence of other mutations like H63D or S65C.

1. *PCR and restriction digest.*
PCR was performed with $5\,\mu l$ DNA using $5\,\mu l$ (50 pmol) each of sense primer 5′-TGGCAAGGGTAAACAGATCC-3′ (Feder *et al*[3]) and antisense primer 5′-TACCTCCTCAGGCAC TCCTC-3′ (Jeffrey *et al*[12]), $24.7\,\mu l$ H_2O, $5\,\mu l$ $10\times$ PCR-buffer, $5\,\mu l$ dNTPs ($2\,\text{mM}$) and $0.3\,\mu l$ *Taq*-Polymerase (Qiagen, Hilden Germany). Cycling conditions were 5 min 94°C, 32 cycles of 1 min at 94°C, 1 min at 58°C and 1.5 min at 72°C, followed by a final incubation of 5 min at 72°C. PCR products were digested with *Rsa*I and visualized on ethidiumbromide-stained 2% agarose gels. The G to A transition at nucleotide 845 (C282Y) creates a new cleavage site in the PCR product.

2. Reverse allele-specific oligonucleotide hybridisation

Two different allele-specific oligonucleotide (ASO) methods (2A and 2B) were used. Both methods required PCR and hybridization of the PCR products on oligonucleotide probes bound on strips.

Method 2A was performed using an LBP-prototype kit (Labo-Biochemical-Products B.V., Netherlands), obtained by Innogenetics N.V., Gent, Belgium. DNA (5 μl) was added to 45 μl of an *HFE* PCR mix and amplified under the following conditions: 9 min 95°C, 40 cycles of 45 s at 95°C, 45 s at 60°C and 1 min at 72°C, followed by a final incubation of 4 min at 72°C. The automated reverse hybridization procedure was performed in an AUTO-LIPA apparatus (Innogenetics N.V., Gent, Belgium) and consisted of hybridization, stringent washes and colour development, using a protocol and chemicals as provided by Innogenetics. Presence of wild type and/or mutation(s) was shown by colour signals on the test strip. The strips contained oligonucleotides specific for the C282Y, H63D and S65C mutations, as well as for the corresponding wild types and a conjugation control.

Method 2B was the GenoType® Hereditäre Hämochromatose-Kit (Hain Lifescience GmbH, Nehren, Germany). PCR with 5 μl DNA, 35 μl primer mix (including dNTPs), 2.7 μl H$_2$O, 2 μl MgCl$_2$, 5 μl 10 × PCR-buffer and 0.3 μl *Taq*-Polymerase (HotStar Taq, Qiagen) consisted of 15 min 95°C, 10 cycles of 30 s at 95°C, 40 s at 58°C, 40 s at 70°C and 25 cycles of 25 s at 95°C, 40 s at 53°C, 40 s at 70°C, followed by 8 min at 70°C. Hybridization, washing and colour detection was performed on a Tecan profiblot apparatus (Tecan Deutschland GmbH, Crailsheim), using the protocol and chemicals as supplied by Hain Liefescience. The strips contained oligonucleotides specific for the C282Y and H63D mutations, as well as for the corresponding wild types and conjugation, specificity and sensitivity controls.

3. Solid-phase oligonucleotide ligation assay (SPOLA)

Method 3 was carried out using the Genespector® *HFE* kit (Variom Biotechnology AG, Berlin, Germany). For PCR, 5 μl DNA was amplified with 12.5 μl primer mix, 2.5 μl dNTP, 2.25 μl H$_2$O, 2.5 μl buffer and 0.25 μl *Taq*-Polymerase for 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, followed by a final incubation of 5 min at 72°C.

The PCR products were added to allele-specific detection probes (for wild type or C282Y) that were covalently linked to 96-well microplates, which in addition contained free common signal probes. Hybridization, ligation, washing and colour labelling was performed using the corresponding chemicals according to the supplied protocol. Presence of the wild type and/or mutant sequence was indicated by a colour signal in the well.

4. Microarray (DNA-chip)

A prototype *HFE* chip assay was provided by BioChip Technologies GmbH, Freiburg, Germany. PCR was performed with 2.5 μl DNA, 1.88 μl primer mix, 3.75 μl dNTPs including Biotin-dUTP, 11.62 μl H$_2$O, 2.5 μl MgCl$_2$, 2.5 μl 10 × PCR-buffer and 0.25 μl *Taq*-Polymerase under the following conditions: 15 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 63°C and 30 s at 72°C, finally 5 min at 72°C.

DNA chips (glass plates spotted with oligonucleotide probes for mutations C282Y, H63D and S65C and the corresponding wild-type probes) were hybridized with the PCR products, washed and stained using the provided chemicals according to the suppliers protocol. Detection of hybridization signals was finally achieved with a BioDetect Reader (GeneScan Europe, Freiburg, Germany).

Statistical methods

Statistical evaluations (χ^2 test, Fisher's exact test, *t*-test, ANOVA, logistic regression analysis, Pearson correlation) were performed using the SPSS package version 11.5. (SPSS Inc., Chicago, USA). In all, 95% confidence intervals (CI) were determined using the program StatXact version 6.0 (Cytel Software Corp., Cambrigde, USA).

Results

General

Between January 2001 and August 2002, 5882 KKH insurants contacted the Institute of Human Genetics of the MHH and requested information on the pilot project and test material. Capillary blood samples were obtained from 3961 (67.3%) of these individuals (Table 1). In 100 cases, new blood samples had to be requested because of test failure, which was mostly due to sparse blood drops and occurred independently of the analytic method used. Of these 100 individuals, 69 provided a second blood

Table 1 Participants of the pilot project on DNA-based HH screening in Germany

Individuals requesting information			Individuals requesting testing					
Sex	Number	%	Sex	Number	%	Age (mean)	Age (median)	Age (range)
Female	3926	66.7	Female	2650	66.9	48.25	48	18–86
Male	1952	33.1	Male	1311	33.1	52.78	55	18–87
Unknown	4	0.1						
All	5882	100	All	3961	100	49.75	51	18–87

5882 Insurants of the KKH requested information from the MHH via telephone, Fax or mail. 3961 blood samples were sent for genetic testing.

sample, which was successfully tested in all cases. Thus, 3930 (99.2%) test results were communicated to the referring doctors.

Of the successfully tested individuals, 67 (1.7%, approximately 1 in 59) were homozygous for C282Y (45 females, 22 males; 67.2 *versus* 32.8%), 485 (12.3%, approximately one in eight) individuals were heterozygous for C282Y (318 females, 167 males; 65.6 *versus* 34.4%), and 3378 (86.0%) of the tested persons did not carry the C282Y mutation (2266 females, 1112 males; 67.1 *versus* 32.9%).

The gender distribution of the individuals requesting information (female 66.7%, male 33.1%) was equal to those who finally requested testing (female 66.9%, male 33.1%). Hence, the uptake rate was 0.675 (2650/3926) in females and 0.672 (1311/1952) in males, which was statistically not different ($P = 0.9$; χ^2 test). However, if the gender distribution of the study participants was compared to those among adult insurants of the KKH (female 63%, male 37%), a statistically significant difference was observed ($P < 0.001$; χ^2 test).

The age distribution among those individuals who requested testing was shifted towards a higher mean and median age of the male participants (Table 1), a difference which was statistically significant ($P < 0.001$; *t*-test).

Technical performance

Every blood sample was tested with two different test methods (Table 2). In 3911 cases, both methods gave concordant results, which were forwarded to the referring doctors. In 19 cases, discordant test results were obtained, and the tests were initially repeated with the same methods. These repeated analyses revealed concordant results in each case, which were then confirmed by a third method. Finally, the confirmed results were sent to the referring doctors. In 11 of these 19 cases, one of the two initial methods resulted in the incorrect diagnosis of heterozygosity instead of homozygosity for the wild type. This error was due to background signals for the mutant probe in nine cases (7 times method 2A, once methods 3 and 4) and to unspecific PCR amplification, leading to the presence of an extra band in both errors of method 1. In four cases, heterozygosity for C282Y was misinterpreted as homozygosity for wild type, due to missing signals for the mutated alleles in presence of the wild type (twice in methods 2A and 4, each). In the remaining four cases, background signals for the wild type led to the misinterpretation of heterozygosity instead of homozygosity for C282Y (3 times in method 3, once in method 4).

Of 7860 tests performed, 7841 (99.76%) gave correct results, assuming that the results were correct if concordant in two different methods. The overall error rate was 0.24% (95% CI: 0.15–0.38%) (Table 2).

The analytic specificity of the test methods with respect to the detection of homozygosity for C282Y was 100%, since not a single case was incorrectly assigned to be homozygous (7726 of 7726 nonhomozygous test challenges, 95% CI: 99.95–100%). Among the 67 homozygotes, 130 of 134 test results were correct. Hence, the overall analytic sensitivity of the test methods with respect to homozygosity was 97% (130/134, 95% CI: 92.5–99.2%).

Costs

Our cost calculations were based on the workload and hands-on time experienced in the pilot project. Depending on the test method used, 9000 to 9600 tests could be performed per year if one full-time technician, one half-time scientist/physician and one half-time secretary perform an *HFE* screening program consisting of receiving

Table 2 Validity of the different test methods

Method	Number of tests	Correct results	Errors	Error rate (%)	P-value*	Number and wrong genotypes (correct genotype, number and second method)
1: RD	2693	2691	2	0.07		**2 het** (wt , 2 2B:ASO)
2A: ASO	1992	1983	9	0.45	*0.0095*	**7 het** (wt, 5 4:Chip, 1 1:RD, 1 3:SPOLA) **2 wt** (het, 2 4:Chip)
2B: ASO	667	667	0	0	0.064	
3: SPOLA	1614	1610	4	0.25	0.15	**3 het** (homo, 2 2A:ASO, 1 1:RD) **1 het** (wt, 1 2A:ASO)
4: Chip	894	890	4	0.45	*0.037*	**2 wt** (het, 2 2A:ASO) **1 het** (wt, 1 2A:ASO) **1 het** (homo, 1 2A:ASO)
Total	7860	7841	19	0.24		

Number of tests refers to the number of single tests. Each individual was tested with two independent test methods. Correctness of results was assumed if both tests gave concordant results. Error refers to the number of incorrect first-testing results as determined by retesting of samples with discordant results. At retesting, both methods gave concordant results in all 19 cases, which were further confirmed with a third method. Error rate refers to the percentage of misinterpreted results per method. Methods: 1 = PCR and restriction digest (RD), 2A and 2B = reverse allele-specific oligonucleotide hybridization (ASO), 3 = solid-phase oligonucleotide ligation assay (SPOLA), 4 = DNA-chip.*P-values were calculated by Fisher's exact test and refer to statistical differences in the error rate when compared to the standard method (method 1). Significant differences ($P < 0.05$) are given in italics. Last column: number and incorrectly obtained results are given in bold (het = heterozygosity for mutation C282Y, wt = wild type for C282Y, homo = homozygosity for C282Y). The correct genotypes are given in parantheses, followed by the number of tests that were performed initially with the given second method.

Table 3 Evaluation of costs per method

	1: PCR/RD	2A: ASO	2B: ASO	3: SPOLA	4: Chip
Samples per day	48	45	45	48	48
Samples per year	9600	9000	9000	9600	9600
Personnel/test (€)	9.60	10.25	10.25	9.60	9.60
Kit/test (€)[a]	0.30	4.80	4.80	2.89	4.80
Filter paper/test (€)	0.20	0.20	0.20	0.20	0.20
Postage/test (€)	1.10	1.10	1.10	1.10	1.10
Costs/test (€)	11.20	16.35	16.35	13.79	15.70

Samples per day denotes to the number of samples that could be processed by the given personnel (one full-time technician, one half-time physician and one half-time secretary). Samples per year denotes the number of samples that could be processed in 200 working days. Personnel costs were derived by dividing the total annual costs for personnel (92.200 Euro) by the number of samples per year. Costs per kit were as would be requested by the supplier assuming an annual test volume of 9000 (9600) samples. Filter paper would cost approximately 20 cents per test (as deduced from newborn-screening costs). Postage = postage for sending an information leaflet, filter paper and a prepaid return envelope to the participants. Methods: 1 = PCR and restriction digest, 2A and 2B = reverse allele-specific oligonucleotide hybridization, 3 = solid-phase oligonucleotide ligation assay; 4 = DNA-chip.
[a]Costs according to the supplier of the commercially available test kits (methods 2B, 3), resp. as deduced from these costs (methods 2A, 4). Method 1: Costs for restriction enzyme and *Taq*-polymerase.

requests for information, sending out information leaflets and test material, providing information by telephone, offering monthly information meetings, receiving and documenting test samples, performing one test per sample, reporting test results to the referring doctors, interpretation of positive test results to the homozygotes by telephone. The average annual costs (Germany) for personnel are as follows: full-time technician: 43.100 €/year; half-time scientist/physician: 30.600 €/year, half-time secretary: 18.500 €/year. The total costs for personnel add up to 92.200 €/year. By dividing the costs for personnel through the estimated annual test numbers, and by adding the costs per test for chemicals and postage, we calculated the overall direct costs per test for each of the five methods to range between 11.20 and 16.35 € (Table 3). These costs do not include pretest consultation (usually done by the GP), posttest counselling (preferably done by a geneticist), clinical assessment by a specialist (e.g. gastroenterologist) and overhead costs such as rent and equipment.

Clinical relevance
Clinical data could be retrieved from 61 (42 females, 19 males) of the 67 homozygotes. In all, 26 (42.6%) of these individuals (13 males, 13 females) already knew their clinical diagnosis of haemochromatosis before sending the blood sample. Of the 61 homozygotes (57.4%), 35 did not know that they were suffering from haemochromatosis (or, at least, were carrying the haemochromatosis genotype) themselves, but in seven of these cases, it was known that family members had clinical evidence of haemochromatosis (four times brothers, once the mother, once the daughter and once the husband). One (female) of the 35 individuals without prior knowledge of haemochromatosis was already treated by phlebotomy because of porphyria cutanea tarda. Hence, 34 newly diagnosed homozygotes (28 females, six males) had never been treated by

phlebotomy. Of these individuals, 11 (32.4%) had normal or borderline ferritin levels (group 1; Table 4), while 23 of these 34 individuals (67.6%) exhibited iron accumulation (groups 2 and 3; Table 4). Among these 23 individuals, eight individuals (five females, three males; group 3) exhibited clinical symptoms and/or signs of organ involvement consistent with haemochromatosis (Table 5). None of these individuals presented with the full clinical picture of severe haemochromatosis with irreversible organ damage.

The median age tended to be higher in those individuals with iron accumulation (53 years, group 2; 57 years, group 3) than in those with normal or borderline ferritin levels (40 years, group 1) (Figure 1); however, the age distribution was not statistically significant between the three groups ($P = 0.2$, ANOVA). Also, no statistically significant differences in sex distribution were observed between the three groups ($P = 0.8$, Fisher's exact test), although the male-to-female ratio was higher in individuals with iron accumulation ($3/12 = 0.25$ in group 2 and $3/5 = 0.6$ in group 3) than in group 1 ($0/11 = 0.0$). When we retrospectively calculated the power of this statistical analysis, we found that the power to detect differences in the age or sex distributions among individuals with iron accumulation would be only 30 and 10%, respectively, which was due to the small numbers of probands.

By performing a logistic regression analysis, we found that the risk for iron accumulation among C282Y homozygotes is neither predictable by gender ($P = 0.062$) nor age ($P = 0.067$). However, if we divided the group of C282Y homozygotes in those >50 years and $\leqslant 50$ years, we found a statistical significant correlation between age (>50 years) and iron accumulation (groups 2 and 3) ($P = 0.026$, Fisher's exact test, odds ratio 8.438, 95% CI 1.457–48.851).

Generally, a significant correlation between ferritin value and age was seen ($P<0.01$; Pearson correlation). Such a correlation is independently present in males and females

Table 4 Laboratory parameters of 34 newly diagnosed C282Y homozygotes

No.	Sex	Age (years)	Ferritin (ng/dl)	Iron (ìg/dl)	TS (%)	AST (U/l)	ALT (U/l)	Group
830	F	37	207	226	ND	8	10	1
216	F	40	194	253	43	ND	ND	1
3515	F	47	187	ND	ND	ND	ND	1
3147	F	40	51	141	ND	8	7	1
3612	F	35	190	ND	ND	ND	ND	1
1157	F	50	204	204	39	32	34	1
6055	F	31	31	154	52	8	11	1
4059	F	32	153	153	51.7	9	7	1
5042	F	49	104	192	75.6	15	8	1
1421	F	67	200	168	72	18	18	1
5531	F	26	41	297	111	9	7	1
1013	F	53	579	170	77	7	8	2
4202	F	46	611	175	99	9	13	2
5493	M	20	278	180	ND	12	12	2
5933	F	57	328	262	ND	11	10	2
6031	F	51	350	175	ND	ND	ND	2
313	M	61	674	198	83	11	13	2
5047	F	37	326	178	88.8	ND	ND	2
4463	F	42	314	217	58	10	15	2
3572	F	67	987	237	ND	16	9	2
780	M	63	718	183	95.4	11	14	2
5829	F	33	373	186	ND	21	14	2
5885	F	55	502	128	ND	10	7	2
1128	F	38	288	243	72	9	5	2
3652	F	67	516	217	92.7	11	14	2
68	F	64	375	232	ND	8	11	2
3267	M	57	795	275	93.9	10	12	3
62	F	33	399	177	96.6	9	6	3
5052	F	57	493	149	63	9	13	3
3831	F	20	279	258	89.5	12	6	3
4537	M	57	1070	214	108	9	13	3
1174	F	61	928	208	ND	13	29	3
1647	F	61	2670	276	93	33	52	3
5143	M	60	2982	250	ND	30	62	3

Age refers to the age at time of testing. Group 1: individuals without iron accumulation (normal (<200 ng/dl) or borderline ferritin). Group 2: individuals with iron accumulation [elevated ferritin and transferrin saturation (TS, $>45\%$) without further laboratory or clinical findings that were consistent with haemochromatosis]. Group 3: Individuals with iron accumulation and further laboratory (ie aspartate aminotransferase (AST) >15 U/l, alanine aminotransferase (ALT >17 U/l)) or clinical findings that were consistent with haemochromatosis. ND: not determined.

(Figure 2), although it is significant in the females, only ($P<0.05$, Pearson correlation). No such correlation was observed between transferrin saturation and age (Figure 3, $P = 0.49$ for males and 0.85 for females, Pearson correlation).

Discussion

Although HH is a candidate for the introduction of a population-based genetic screening due to its frequent occurrence, the simple diagnostic procedure and the availability of a highly effective treatment, several important issues still have to be addressed in pilot studies.[4] We aimed at obtaining more information on some of these issues and initiated a nationwide pilot project on DNA-based HH screening.

Since approximately 90% of the German HH patients are homozygous for the mutation C282Y, we routinely tested only for this particular mutation. The presence of other mutations (ie H63D and S65C) was not evaluated in this pilot project, because the clinical relevance of these mutations is far less established than those of C282Y. Mainly because of the high detection rate of C282Y testing and the unclear relevance of other mutations, we feel that genetic HH screening should focus on testing C282Y only. However, we offered further testing of H63D to C282Y heterozygotes, either when personally requested during counselling, or when requested by the GP because of medical reasons.

One of the issues which is not conclusively investigated to date is the analytic validity of genetic testing in population screening. Palomaki *et al*[13] analysed published results of the Molecular Genetics Survey (external profi-

Table 5 Clinical signs and symptoms of the eight individuals of group 3 (Table 4)

No.	Symptoms and clinical signs	Family history	Phlebotomy[a]
3267	Abdominal fullness, nausea, fatigue, abdominal pain, arthralgia; Ultrasound: liver disease; abdominal CT scan: slightly enlarged liver, small hypodense lesion; liver histology: high-grade parenchymal siderosis	Father died of liver carcinoma	Yes
62	Weakness, fatigue, loss of libido, arrhythmia; skin pigmentation; Ultrasound: hepatomegaly, splenomegaly	None	Yes
5052	Depressive moods, arthralgia; skin pigmentation	Mother died of liver disease	Unknown
3831	Weakness, fatigue, lethargy, arthralgia	One younger sister was subsequently found to have elevated ferritin values and homozygosity for C282Y	Yes
4537	Loss of libido, psychosis, diabetes mellitus, elevated urea; Ultrasound: enhanced hepatic echogenicity, no evidence of cirrhosis	One nephew and one niece were subsequently found to have elevated ferritin values and homozygosity for C282Y	Yes
1174	Weakness, fatigue, abdominal pain, arthralgia, borderline hyperglycaemia; Ultrasound: liver slightly enlarged, enhanced echogeneity	None	Yes
1647	Arrhythmia, diabetes mellitus; Ultrasound: normal liver	None	Yes
5143	Diabetes mellitus; Ultrasound: liver size normal, markedly increased echogenicity of the parenchyma	Unknown	Yes

Data were retrieved from medical records, questionnaires and interviews.
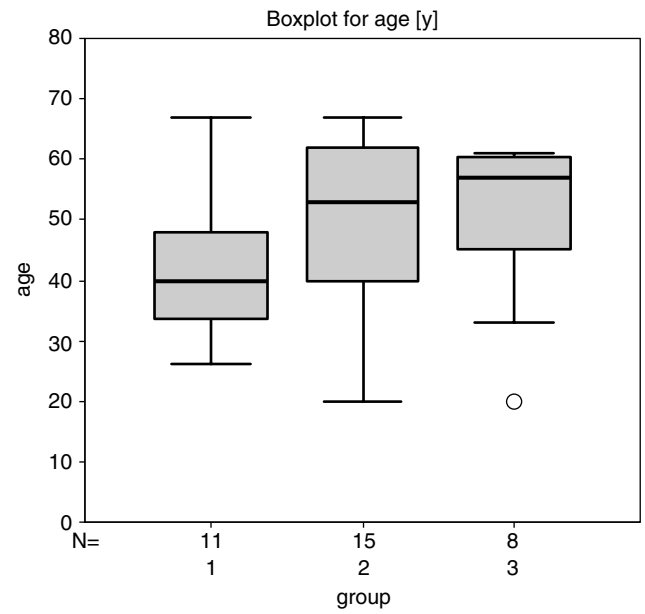aThe amount of iron removed via phlebotomy is unknown.

Figure 1 Boxplot for age. The ages (y-axis) of 34 newly diagnosed C282Y homozygous individuals are plotted by group (x-axis; group 1 (individuals without iron accumulation): $n = 11$, group 2 (healthy individuals with iron accumulation): $n = 15$, group 3 (individuals with iron accumulation and further laboratory or clinical findings consistent with haemochromatosis): $n = 8$). Thin lines: age range (note: one individual of group 3 lies outside the range and is represented by a circle). Boxes: 25 to 75% range. Vertical line: median age within group.

ciency testing for HFE mutations) performed by the American College of Medical Genetics/College of American Pathologists (ACMG/CAP) between 1998 and 2002 and estimated the analytic sensitivity and specificity for C282Y homozygosity to be 98.4 and 99.8%, respectively.[13] This study, however, explored the test performance in the theoretical context of population screening and did not take into account the different test methods used. We tested all samples in a real diagnostic setting with two independent test methods and were thereby able to compare the robustness of the technical performance including the interpretation of the test results of five different test systems. The assay robustness is one major component of the analytic validity of genetic testing. Assuming that the test results were correct if both methods revealed the same results, we obtained error rates between 0 and 0.45%. Although the error rates of two of the methods were significantly higher than the error rate of our standard laboratory method for C282Y mutation testing (PCR and restriction enzyme), we have to take into account that the personnel was more experienced to use the standard method and that fewer errors occurred after gaining more experience with the other methods. This
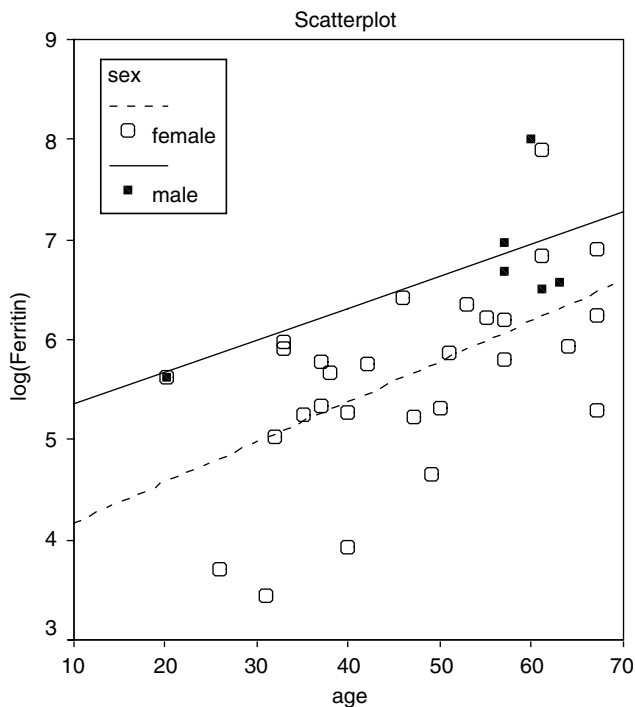
Scatterplot

Figure 2 Scatterplot for ferritin (logarithmic values for ng/dl, *y*-axis) *versus* age (years, *x*-axis) of homozygous individuals. Males are represented by filled squares, females by circles. Solid line: regression line for males. Dotted line: regression line for females.
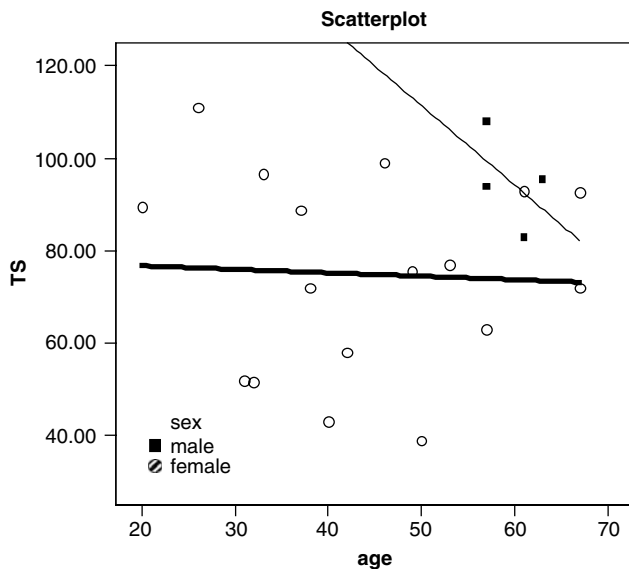
Scatterplot

Figure 3 Scatterplot for transferrin saturation (TS, given in %, *y*-axis) *versus* age (years, *x*-axis) of homozygous individuals. Males are represented by filled squares, females by circles. Solid line: regression line for males. Dotted line: regression line for females.

learning effect becomes evident when the error rates of both ASO methods (0.45%, method 2A *versus* 0%, method 2B) were compared under the aspect that the latter tests were performed after the former tests had been completed, and that the technical performance of both tests was similar. The overall sensitivity and specificity of the test methods was 97 and 100%, respectively. However, although these rates reflect the high robustness and reproducibility of the technical performance, they cannot be equated with an overall analytic sensitivity and specificity since they do not take into account possible laboratory errors like sample mix-up or other clerical errors. To assess these types of errors, it would be necessary to test two or more independent blood samples from each individual.

Several cost/benefit studies had been performed to investigate whether genetic and/or biochemical HH screening will be acceptable at the population level.[9–11,14–16] Most of these studies provided good economical arguments for the introduction of HH population screening. However, to the best of our knowledge, none of these studies had actively assessed the direct laboratory costs of genetic HH testing, which occur when a mass screening is performed. One of the aims of our pilot study was the assessment of such costs. Only slight differences in these costs were attributable to the test method employed. We based our calculations on the assumption that between 9000 and 9600 individual tests could be performed per year by a team consisting of one full-time technician, one half-time scientist/physician and one half-time secretary. The capacity per personnel would presumably be higher and the total laboratory costs per test would slightly decline in a mass screening of a much larger number of individuals. But even if it is possible to decrease the costs per test substantially (e.g. to a range of 5 to 10 €), it has to be taken into account that the laboratory costs are only one cost factor and that other costs such as those for pre- and posttest councelling will also have to be considered in future cost/benefit calculations.

One of the most controversial issues regarding HH screening pertains to its clinical relevance. Especially the controversy on the disease penetrance is still a matter of ongoing discussion.[17–23] If restricted to the full clinical picture of haemochromatosis, the penetrance may be as low as less than 1[24] or 2%[25]. If haemochromatosis is defined as iron accumulation in conjunction with clinical findings that were consistent with haemochromatosis, the penetrance was as high as 50[26] to 52%[27]. These different figures reflect the variable expression of a common disease, which, for presently unknown reasons, is rarely seen in its full clinical expression. Following this consideration, eight of 34 (23.5%) newly diagnosed C282Y homozygous individuals could be classified as being affected with HH. Iron accumulation without further signs or symptoms was present in 15 homozygous individuals. Hence, although

iron accumulation was present in 21 of 34 homozygous individuals (61.8%), only 28.6% of these 21 individuals showed additional clinical or laboratory findings at the time of screening. Considering the positive correlation between age and ferritin level (Figure 2), it can be assumed that many of the individuals with currently normal or borderline ferritin levels will develop iron overload later in life. Age and sex distribution of the newly diagnosed C282Y homozygous individuals point towards a higher risk for iron accumulation in male and older individuals.

No positive correlation was seen between age and transferrin saturation (TS). In fact, the highest value for TS was seen in a 26-year-old female individual with a normal ferritin level. Since the elevation of TS precedes the elevation of ferritin, almost all of the C282Y homozygous individuals presented with an elevated TS. TS measurement, therefore, could be an early predictor for the C282Y homozygous genotype, rather than for the development of HH. This requires analyses in larger patient cohorts.

The results concerning the clinical relevance of this pilot study must be seen in the context of a very high rate (1.7%) of individuals homozygous for C282Y among those who requested testing. Partially, this high rate can be explained by the fact that 42.6% of these already knew their clinical diagnosis of haemochromatosis before sending the blood sample. These individuals used the pilot project for an independent confirmation of the diagnosis and to obtain more information about the clinical course and the inheritance of this disease. However, at least 34 of the C282Y homozygous individuals (0.86% = 1/115 of the tested persons, $n = 3930$) were not aware of their haemochromatosis genotype. Assuming heterozygosity rates of one in eight (as seen in this study), one in 10 (as determined by testing healthy blood donors, unpublished results) or one in 13[28] in Germany, homozygosity rates of 1/256, 1/400 or 1/657 would be expected. By stratifying for gender and removing those 35 individuals (13 males, 14 females) with a known diagnosis, we observed a homozygosity rate of 1/214 among males (eight of 1288) and 1/93 among females (28 of 2615). We assume that the overrepresentation of newly detected homozygous individuals (particular among females) indicates a bias in the study population towards individuals who were concerned about their health, either because of personal unexplained laboratory parameters or subtle symptoms, or because of a family history of disease. The apparent difference in gender distribution may indicate to a greater concern about health issues among females than among males. The assumption of a bias in the study population is also supported by the observation that the study participants have a statistically significant different gender distribution (higher rate of females) than the 1.4 million KKH insurants. Presumably, the proportion of male insurants who read the KKH journal is lower than the proportion of female readers. Alterna-

tively, fewer males are interested in participating in such a study because of less health concern than females. Interestingly, the age distribution of male and female participants was significantly different. Presumably, older males have more time and/or interest to read the KKH Journal, or to take part in such a study.

The study is not aiming to represent a general population screening, as the study population is not representative for the German population (as exemplified by the high rate of female insurants of the KKH, compared to the general population). Additionally, there is a bias related with the system used to select participants (active by insurants and not random). Therefore, the data relating to the prevalence of the mutation are not reflecting the situation in the general population, and it is not adequate to calculate directly the penetrance of the disease in the study population. Moreover, only a small minority of the newly diagnosed homozygotes was investigated with invasive methods like liver biopsy. Likewise, the study was not designed to obtain precise data on the incidence of the disease, the way to perform the test or to reach a final conclusion whether to screen or not to screen for HH in the general population. However, we were able to gain important novel data particular on the technical performance and on the costs and the clinical relevance of genetic screening for HH.

Despite the obvious biases mentioned above, we can summarize that we were able to identify a relatively high number of individuals with haemochromatosis or at risk for this disorder by testing a relatively small number of persons. We assume that preferentially those persons who are for whatever reasons concerned to be affected by, or to be at risk for haemochromatosis, will participate in an HH screening program, thereby reducing the cost per preventable case of clinical haemochromatosis. Such a program can be performed at reasonable laboratory costs. The test methods for C282Y mutation detection are robust, highly sensitive and specific.

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