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Original Article

Impact of *HFE* gene variants on iron overload, overall survival and leukemia-free survival in myelodysplastic syndromes

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Abstract: Although iron overload is a clinical challenge, little is known about the clinical impact of *HFE*-variants in myelodysplastic syndromes (MDS) to date. We analyzed the *HFE* status in 167 MDS patients and 494 healthy controls. One or more of the 3 *HFE*-variants (H63D, C282Y, S65C) were found in 65/167 (38.9%) MDS patients and in 164/494 (33.2%) controls. At diagnosis, the median serum ferritin levels were higher in MDS patients with *HFE*-variants (409 µg/L; range: 23-7415) compared to those without *HFE*-variants (346.5 µg/L; range: 10-5450) (P=0.62). Moreover, '*HFE*-mutated' patients had a slightly faster increase in serum ferritin in follow up examinations. The percentage of patients with *HFE*-variants was higher in refractory anemia (RA) (22/53=41.5%) or RA with ring sideroblasts (RARS) (17/39=43.6%) compared to RA with excess of blasts (RAEB) (16/46=34.8%) or RAEB in transformation (RAEB-T) (5/17=29.4%). Differences were also detectable when comparing low- and high-risk MDS variants defined by the World Health Organization classification. There was no significant correlation between *HFE*-variants and MDS-related somatic mutations. Progression-free survival was substantially longer in patients with *HFE*-variants compared to those without *HFE*-variants H63D and C282Y (P=0.089). Together, the *HFE*-variants H63D and C282Y are frequently detected in Austrian MDS patients. These patients have substantially higher ferritin levels at diagnosis, accumulate iron slightly faster and have a better progression-free survival than non-mutated patients.

Keywords: MDS, ferritin, iron overload, *HFE* gene variants, iron chelation, NGS

Introduction

Myelodysplastic syndromes (MDS) are a heterogeneous group of myeloid neoplasms found most commonly in patients aged >60 years [1-4]. Clinical features of MDS include an abnormal differentiation and maturation of myeloid precursor cells, bone marrow (BM) failure with peripheral blood cytopenia, and a genetic instability with enhanced risk of progression to secondary acute myeloid leukemia (AML) [1-4]. The classification of MDS is based on morphologic and molecular criteria provided by the World Health Organization (WHO) and the French-American-British (FAB) cooperative study group [4-9]. However, in each diagnostic category, the course of disease and prognosis of patients vary. During the past decades a number of attempts have been made to establish useful scoring systems predicting survival and/or AML evolution [9-15]. These scoring systems are based on multiple prognostically important

parameters, including the percentage of BM blasts and the number of peripheral cytopenias. The 'International Prognostic Scoring System' (IPSS) has been established in 1997 [15]. This scoring system is based on the percentage of BM blasts, number of cytopenias, and chromosomal abnormalities [15]. To improve the prognostic power, the IPPS score has recently been revised into the IPSS-R [16].

However, apart from WHO-related or IPSS-based variables, other prognostic parameters (not included in the IPSS or IPSS-R) have also been identified [9, 13, 17-19]. A number of previous and more recent studies have shown that an elevated serum lactate dehydrogenase (LDH) is associated with a poor prognosis in MDS [13, 17-19]. Other studies have shown that age at diagnosis, the transfusion frequency, comorbidities, and elevated serum ferritin levels are associated with a poor survival in MDS [20-26]. Within the last 10 years, molecular markers have evolved to play a substantial role in the prognosis and evolution of MDS [9-27]. However, such molecular markers were also detected in the absence of an overt malignancy. When detected in healthy individuals these mutations are termed clonal hematopoiesis with indeterminate potential (CHIP) [27-31]. In many cases of CHIP, hematopoiesis remains normal over years or even decades. However, some of these mutations exhibit a higher risk and were thus also called clonal hematopoiesis with oncogenic potential (CHOP) [27].

Patients with MDS are often chronically transfused with significant transfusion-related morbidity. This is in part a result of chronic iron overload and reflected by high serum ferritin levels [25, 26, 32]. Other studies have shown that the susceptibility for iron uptake and iron overload in various tissues is determined by genetic factors and may increase with age [33, 34]. One major regulator of iron uptake and metabolism is the *HFE* gene [34, 35]. The *HFE* protein is involved in the regulation of hepcidin expression and secretion. In patients with *HFE* mutations, the interaction of *HFE* with transferrin receptors TFR1 and TFR2 is disrupted, resulting in decreased hepcidin expression which in turn is responsible for an increased iron uptake in target cells and thus iron overload [34, 35].

The *HFE* gene variants (some also refer to these as mutations or single nucleotide polymorphism) H63D and C282Y are considered to be critically involved in iron overload. Interestingly, these variants were reported to be more frequently present in patients with MDS compared to healthy controls or patients with other hematologic neoplasms [36-40]. However, the number of cases examined in these studies was small, the exact incidence of these variants remains unknown, and so far, little is known about the influence of these genetic factors on survival or disease-progression in MDS.

In this study we determined the prevalence and impact of *HFE* gene variants on survival and AML-development in Austrian patients with MDS. Our data confirm previous studies suggesting that the *HFE*-variants H63D and C282Y can be detected frequently in patients with MDS. These *HFE*-variants may predispose for rapid iron overload in chronically transfused patients, but massive iron overload also occurs in those who do not have a *HFE* mutation. Finally, we show that the *HFE* status correlates with the prognosis and survival in MDS.

Patients and methods

Patients

A total number of 167 unselected patients with MDS (females, n=79; males, n=88; median age: 69 years; range: 28-84 years) were examined retrospectively for the presence of *HFE* gene variants in our center (observation period: 1992-2019). All patients gave their written consent before blood or bone marrow cells were analyzed. The study was approved by the ethics committee of the Medical University of Vienna (number: 402/2007). The patient's characteristics are shown in **Tables 1** and **2**. All samples were collected during routine investigations. Patients were classified according to FAB cooperative study group criteria and WHO 2016 criteria. Moreover, samples from 494 healthy donors were collected as control cohort. All patients and controls were of Austrian origin, and all samples were collected at the Medical University of Vienna.

Evaluation of disease-related parameters at diagnosis and in the follow-up

The following blood parameters were recorded at diagnosis: complete blood count including

Table 1. Patient characteristics according to FAB classification

FAB-Subtype	Patients n (%)	WBC (G/L) median (range)	Hb (g/dL) median (range)	Plt (G/L) median (range)	Ferritin (µg/L) median (range)	LDH (U/L) median (range)
RA	53 (31.5)	3.44 (1.7-10.2)	9.5 (5.6-13.3)	115 (3-898)	368 (42-2300)	209 (127-1751)
RARS	39 (15.4)	5.04 (1.19-12.11)	9.4 (5.9-11.4)	199 (31-807)	586.5 (23-7415)	176 (103-457)
RAEB	47 (27.9)	2.88 (0.63-76.5)	9.1 (6.8-13.6)	89 (13-398)	287.5 (36-1701)	235 (147-1059)
RAEB-T	17 (10.1)	2.99 (0.58-25.38)	8.9 (7.2-11.4)	100 (25-390)	598 (10-1980)	272 (117-2600)
CMML	12 (7.1)	7.89 (2.83-26.64)	10.5 (5.4-14.2)	92 (3-241)	488.5 (64-2180)	184.5 (137-862)

Abbreviations: FAB, French-American-British; WBC, white blood count; Hb, hemoglobin; Plt, platelets; LDH; lactate dehydrogenase; RA, refractory anemia; RARS, RA with ring sideroblasts; RAEB, RA with excess of blasts; RAEB-T, RAEB in transformation; CMML, chronic myelomonocytic leukemia.

Table 2. Patient Characteristics according to WHO 2016 classification

WHO 2016 Subtype	Patients n (%)	WBC (G/L) median (range)	Hb (g/dL) median (range)	Plt (G/L) median (range)	Ferritin (µg/L) median (range)	LDH (U/L) median (range)
MDS-5q	10 (5.9)	4.84 (2.32-8.26)	9.15 (7.7-11.2)	317 (114-898)	371.5 (42-1390)	178.5 (158-283)
MDS-EB-1	29 (17.2)	3.26 (0.63-76.5)	9.7 (6.8-13.6)	90 (13-398)	263 (36-1170)	245 (147-1059)
MDS-EB-2	28 (16.6)	2.69 (0.58-32)	8.75 (7.1-12.6)	93 (16-390)	294 (10-1980)	225 (144-2600)
MDS-SLD	18 (10.7)	4.12 (1.79-10.2)	10.2 (5.6-12.2)	144 (3-648)	237.5 (96-902)	227.5 (137-369)
MDS-RS-SLD	26 (15.4)	5.05 (1.66-12.11)	9.5 (6.7-11)	236 (31-369)	453 (23-7415)	180 (121-1751)
MDS-MLD	26 (15.4)	3.08 (1.7-6.8)	9.7 (7.3-13.3)	89 (6-295)	538 (43-2300)	203.5 (127-641)
MDS-RS-MLD	12 (7.1)	4.47 (1.19-6.8)	9.3 (5.9-11.4)	133 (64-807)	695 (325-5450)	162 (103-226)
CMML-1	10 (5.9)	7.89 (2.83-18.89)	10.5 (6.7-14.2)	96 (3-241)	488.5 (64-1100)	179 (137-862)
CMML-2	2 (1.1)	16.52 (6.4-26.64)	8.4 (5.4-11.4)	75 (71-80)	1125.5 (71-2180)	335 (301-369)
AML dys.	7 (4.1)	2.99 (1.14-24.37)	10.1 (7.5-11.4)	50 (28-241)	879 (339-1250)	264 (117-395)

Abbreviations: WHO, World Health Organization; WBC, white blood count; Hb, hemoglobin; Plt, platelets; LDH; lactate dehydrogenase; MDS, Myelodysplatic Syndrome; MDS 5q-, MDS with isolated 5q-; MDS-EB1, MDS with excess of blasts-1; MDS-EB2, MDS with excess of blasts-2; MDS-SLD, MDS with single-lineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts (RS) and single-lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-MLD, MDS with RS and multilineage dysplasia; CMML, Chronic Myelomonocytic Leukemia; AML dys., Acute Myeloid Leukemia with myelodysplasia-related changes.

differential count, serum chemistry including serum ferritin, transferrin saturation, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and LDH. In all patients, the transfusion frequency (red cell concentrates per month, recorded in the first 3 years of observation) as well as comorbidity according to the Charlson index and the hematopoietic cell transplantation-specific comorbidity index, were determined [41, 42]. BM investigations included aspirate smears (Wright-Giemsa stains), routine histology and immunohistochemistry, flow cytometry (percent of CD34+ blast cells), and cytogenetics. Chromosome analysis and fluorescence in situ hybridization were performed and reported according to ISCN guidelines [43]. During the follow up, the following parameters were examined (at least every three months): blood counts and differential counts, serum chemistry including LDH, GOT, GPT, ferritin, and transferrin-saturation. The BM was re-examined in case of suspected progression.

Reverse hybridization to detect 18 mutations related to iron overload

In a first step, genomic DNA isolated from the BM of 52 patients with MDS was examined for the presence of mutations in genes relevant to iron uptake or metabolism. DNA samples were screened for 12 mutations in the *HFE* gene (V53M, V59M, H63D, H63H, S65C, Q127H, P160delC, E168Q, E168X, W169X, C282Y, Q283P), 4 mutations in the gene encoding transferrin receptor-2 (*TRF2*, E60X, M172K, Y250X, AVAQ594-597del), and 2 mutations in the gene coding for ferroportin (*FPN1,* N144H, V162del) using the Haemochromatosis StripAssay A (Vienna Lab Labordiagnostika GmbH, Vienna, Austria) as described [44].

Restriction fragment length polymorphism (RFLP) analysis of 3 common mutations (HFE H63D, S65C and C282Y mutation)

Genomic DNA was amplified by polymerase chain reaction (PCR) using PCR primers de-

scribed by Feder et al. [45]. Identification of *HFE* H63D was performed using the restriction enzymes Mbo I. After cleavage with Mbo I, samples without mutations showed two fragments of 138 and 70 base pairs (bp), whereas samples with mutations exhibited an uncleaved PCR fragment of 208 bp. Detection of *HFE* S65C was conducted using the restriction enzymes Hinf I as described [46]. Cleavage with Hinf I in samples without mutation revealed two fragments of 147 and 61 bp, whereas in samples containing *HFE* S65C, the PCR fragment of 208 bp remained uncleaved. The *HFE* C282Y variant was analyzed using the restriction enzymes Rsa I, which revealed two fragments of 249 and 140 bp in samples without mutation and three fragments of 249, 111, and 29 bp in samples containing *HFE* C282Y. All restriction fragments were analyzed by electrophoresis using 6% polyacrylamide gels (Novex, San Diego, CA), followed by Sybr Green staining (Nucleic Acid Gel Stain, Molecular Probe, Eugene, Oregon). In patients with MDS, all three *HFE*-variants were analyzed. By contrast, in healthy controls, only the *HFE*-variants H63D and C282Y were examined because at the time of investigation, no RFLP for the *HFE*-variant S65C was available.

Next generation sequencing (NGS)

Genomic DNA (gDNA) was extracted from peripheral blood (PB) or bone marrow (BM) cells using the QIAsymphony Sp Instrument with the QIAsymphony DNA Midi Kit (Both Qiagen, Hilden, Germany). Quantification of gDNA was performed by fluorometric quantitation using the Qubit dsDNA BR Assay Kit and a Qubit 3.0 Fluorometer (Both Thermo Fisher Scientific, Waltham, USA) and diluted to a final concentration of 200 ng. Library preparation was performed with the Myeloid Solution (Sophia Genetics, Lausanne, Switzerland) according to the manufacturer's instructions. MiSeq System (Illumina, San Diego, California) was used as sequencing platform. Variant calling was performed using the DDM Software (Sophia genetics). Mutations of *ASXL1, SF3B1, SRSF2, TET2, DNMT3A and RUNX1* were classified as clonal hematopoiesis of indeterminate potential (CHIP) and mutations of *IDH2, EZH2, SETBP1, U2AF1, CBL, IDH1, CSF3R, FLT3, JAK2, WT1, ZRSR2, KRAS, NPM1, CALR, MPL, ABL1, BRAF, CEBPA, ETV6, HRAS, KIT, PTPN11* as clonal hematopoiesis with oncogenic potential according to recently published criteria [27].

Statistical evaluations

Differences in the frequency of *HFE*-variants between subgroups of patients and controls were calculated by Chi-square test. The probability of cumulative survival and AML-free survival were calculated by the product limit method of Kaplan and Meier. Differences among various subgroups of patients concerning survival and AML-free survival were determined by log rank test. Differences between *HFE*-variants and clinical and/or laboratory parameters were analyzed by Mann-Whitney-U-test. Correlations between the presence of CHIP/CHOP and absence or presence of *HFE*-variants was assessed by Chi-square test. Concerning the primary endpoint, progression (AML)-free survival, a power analysis revealed that the study has sufficient power of 80% to detect a relevant hazard ratio of 1.6 at the 5% (two-sided) significance level.

Results

Initial screen using the haemochromatosis StripAssay A

In a first step, we applied a broad screen to detect variants of key genes involved in iron uptake and/or metabolism in 52 patients with MDS (data not shown). Because none of these patients showed *TRF2*-, *FPN1*- or rare *HFE* mutations, subsequent analyses were conducted using MDS patient-derived cells and restriction fragment length polymorphism (RFLP) to detect the more common *HFE*-variants H63D, C282Y, and S65C. In our cohort of healthy controls, RFLP analysis of H63D and C282Y was also performed.

Frequency of HFE-variants in patients with MDS and comparison to controls

In 65/167 patients with MDS (38.9%) one or more of the 3 *HFE*-variants (H63D, C282Y, S65C) were detected. The most frequent single *HFE*-variant, H63D, was detected in 50 patients with MDS (29.9%), followed by C282Y (8 patients, 4.8%) and S65C (3 patients, 1.8%) (**Table 3**). Four patients (2.4%) were found to harbor two *HFE* mutations, namely H63D and

Table 3. Distribution of *HFE*-variants in various FAB categories and comparison to controls

FAB subtype	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y	H63D	S65C	Heterozygous*	all variants
RA	3/53 (5.7%)	17/53 (32.1%)	1/53 (1.9%)	1/53 (1.9%)	22/53 (41.5%)
RARS	3/39 (7.7%)	13/39 (33.3%)**	1/39 (2.6%)	0/39 (0%)	17/39 (43.6%)
RAEB	2/46 (4.3%)	12/46 (26.1%)	1/46 (2.2%)	1/46 (2.2%)	16/46 (34.8%)
RAEB-T	0/17 (0%)	4/17 (23.5%)	0/17 (0%)	1/17 (5.9%)	05/17 (29.4%)
CMML	0/12 (0%)	4/12 (33.3%)	0/12 (0%)	1/12 (8.3%)	05/12 (41.7%)
All MDS patients	8/167 (4.8%)	50/167 (29.9%)	3/167 (1.8%)	4/167 (2.4%)	65/167 (38.9%)
Austrian healthy controls	42/494 (8.5%)***	116/494 (23.5%)****	n.d.	6/494 (1.2%)	164/494 (33.2%)

*Heterozygous for *HFE*-variants C282Y and H63D. **1/39 (2.8%) homozygous. ***1/494 (0.2%) homozygous. ****7/494 (1.4%) homozygous. Abbreviations: FAB, French-American-British; RA, refractory anemia; RARS, RA with ring sideroblasts; RAEB, RA with excess of blasts; RAEB-T, RAEB in transformation; CMML, Chronic Myelomonocytic Leukemia; n.d., not detectable.

Table 4. Distribution of *HFE*-variants in various WHO 2016 categories

WHO 2016 subtype	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y	H63D	S65C	Heterozygous*	all variants
MDS 5q-	1/10 (10%)	1/10 (10%)	0/10 (0%)	0/10 (0%)	2/10 (20%)
MDS-EB1	2/29 (6.9%)	10/29 (34.5%)	0/29 (0%)	0/29 (0%)	12/29 (41.4%)
MDS-EB2	0/27 (0%)	3/27 (11.1%)	1/27 (3.7%)	2/27 (7.4%)	6/27 (22.2%)
MDS-SLD	0/26 (0%)	11/26 (42.3%)**	0/26 (0%)	0/26 (5.9%)	11/26 (42.3%)
MDS-RS-SLD	0/18 (0%)	7/18 (38.9%)	0/18 (0%)	0/18 (0%)	7/18 (38.9%)
MDS-MLD	2/13 (15.4%)	9/13 (69.2%)	1/13 (7.7%)	1/13 (7.7%)	13/26 (50%)
MDS-RS-MLD	3/12 (25%)	2/12 (16.7%)	1/12 (8.3%)	0/12 (0%)	6/12 (50%)
CMML1	0/10 (0%)	4/10 (40%)	0/10 (0%)	0/10 (0%)	4/10 (40%)
CMML2	0/2 (0%)	0/2 (0%)	0/2 (0%)	1/2 (50%)	1/2 (50%)
AML dys.	0/7 (0%)	3/7 (42.9%)	0/7 (0%)	0/7 (0%)	3/7 (42.9%)
All MDS patients	8/167 (4.8%)	50/167 (29.9%)	3/167 (1.8%)	4/167 (2.4%)	65/167 (38.9%)

*Heterozygous for *HFE*-variants C282Y and H63D. **1/26 (3.8%) homozygous. Abbreviations: WHO, World Health Organization; MDS, Myelodysplatic Syndrome; MDS 5q-, MDS with isolated 5q-; MDS-EB1, MDS with excess of blasts-1; MDS-EB2, MDS with excess of blasts-2; MDS-SLD, MDS with single-lineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts (RS) and single-lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-MLD, MDS with RS and multilineage dysplasia; CMML, Chronic Myelomonocytic Leukemia; AML dys., Acute Myeloid Leukemia with myelodysplasia-related changes.

C282Y. Only one patient (subtype RARS) was homozygous for *HFE*-variant H63D. In the remaining patients only heterozygous *HFE*-variants were detected. We next compared our MDS patients with the control cohort and found a slightly higher frequency of *HFE*-variants (H63D, C282Y and S65C) in our MDS patients (38.9%) compared to healthy Austrian controls (33.2%) (P=0.18) (**Table 3**).

Frequency of HFE-variants in FAB, WHO, IPSS and IPSS-R subgroups

When examining FAB subgroups of MDS, the percentage of cases with *HFE*-variants was higher in those without blast cell increase (refractory anemia [RA], 41.5%; RA with ring sideroblasts [RARS], 43.6%) compared to patients with advanced MDS (RA with excess of blasts [RAEB], 34.8%; RAEB in transformation [RAEB-T], 29.4%) (**Table 3**). In chronic myelomonocytic leukemia (CMML) 41.7% of the patients had a *HFE*-variant (**Table 3**). Similar differences were also observed between subgroups of MDS patients classified by WHO criteria (**Table 4**). The prevalence of *HFE*-variants was 50.0% in patients with ring sideroblasts (RS) and multilineage dysplasia (MDS-RS-MLD), 50.0% in MDS-MLD, 42.3% in MDS with single-lineage dysplasia (MDS-SLD), 38.9% in MDS with RS and single-lineage dysplasia (MDS-RS-SLD), 41.4% in patients with excess of blasts-1 (MDS-EB1), 22.2% in MDS-EB2, and 20% in MDS with isolated 5q-. These differences in the prevalence of *HFE*-variants among the FAB- or

Table 5. Distribution of *HFE*-variants in IPSS categories

IPSS subgroup	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y	H63D	S65C	Heterozygous*	all variants+
Low risk	1/45 (2.2%)	14/45 (31.1%)	1/45 (2.2%)	1/45 (2.2%)	17/45 (37.8%)
Intermediate risk (all)	7/89 (7.9%)	22/89 (24.7%)**	2/89 (2.2%)	3/89 (3.4%)	34/89 (38.2%)
Intermediate risk-I	4/57 (7%)	17/57 (29.8%)***	1/57 (1.8%)	2/57 (3.5%)	24/57 (42.1%)
Intermediate risk-II	3/32 (9.4%)	5/32 (15.6%)	1/32 (3.1%)	1/32 (3.1%)	10/32 (31.3%)
High risk	0/10 (0%)	2/10 (20.0%)	0/10 (0%)	0/10 (0%)	2/10 (20.0%)

+Due to missing parameters only 145 patients were included for IPSS subgroup analysis. *Heterozygous for *HFE*-variants C282Y and H63D. **1/89 (1.1%) homozygous. ***1/57 (1.8%) homozygous. Abbreviations: IPSS, international prognostic scoring system.

Table 6. Distribution of *HFE*-variants in IPSS-R categories

IPSS-R subgroup	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y	H63D	S65C	Heterozygous*	all variants+
Very Low risk	0/15 (0%)	5/15 (33.3%)	0/15 (0%)	0/15 (0%)	5/15 (33.3%)
Low Risk	4/58 (6.9%)	18/58 (31%)**	2/58 (3.4%)	1/58 (1.7%)	25/58 (43.1%)
Intermediate risk	1/23 (4.3%)	5/23 (21.7%)	0/23 (0.0%)	2/23 (8.7%)	8/23 (34.8%)
High risk	2/31 (6.5%)	8/31 (25.8%)	1/31 (3.2%)	1/31 (3.2%)	12/31 (38.7%)
Very high risk	1/17 (5.9%)	2/17 (11.8%)	0/17 (0%)	0/17 (0%)	3/17 (17.6%)

+Due to missing parameters only 145 patients were included for IPSS-R subgroup analysis. *Heterozygous for *HFE*-variants C282Y and H63D. **1/58 (1.7%) homozygous. Abbreviations: IPSS-R, revised international prognostic scoring system.

WHO-subgroups of MDS or between the various FAB- or WHO subgroups and our healthy controls were not statistically significant.

High or very high risk patients (according to the IPSS or IPSS-R, respectively) were found to have a lower prevalence of *HFE*-variants (IPSS high: 20.0%; IPSS-R very high: 17.6%) compared to the other risk groups (IPSS low: 37.8%, int-1: 42.1%, int-2: 31.3%; IPSS-R very low: 33.3%, low: 43.1%, int: 34.8%, high: 38.7%) (**Tables 5** and **6**). Again, these differences did not reach statistical significance which is best explained by the relatively low number of patients in the high risk groups.

Frequency of the different HFE-variants in FAB, WHO, and IPSS subgroups

In a next step, we analyzed the prevalence of the distinct *HFE* gene variants. The H63D *HFE* gene mutation was found to be expressed most frequently in patients with RARS and CMML (33.3%, each). In the other FAB categories, this *HFE*-variant was also detectable, namely in RA (32.1%), RAEB (26.1%), and RAEB-T (23.5%). In all these MDS variants except for RAEB-T the prevalence of the H63D *HFE*-variant was higher compared to healthy controls (23.5%) (**Table 3**).

The *HFE*-variant C282Y could be detected in all FAB categories except for RAEB-T and CMML (**Table 3**). In RARS patients the frequency of this gene variant was 7.7% and thus comparable to our healthy controls (8.5%). The expression of different *HFE* gene variants in the WHO subgroups is shown in **Table 4**. The prevalence of the three analyzed *HFE*-variants differed also substantially among the IPSS and IPSS-R-groups. High and very high risk patients had a markedly lower prevalence of *HFE*-variants (**Tables 5** and **6**).

Correlations between HFE gene variants and single prognostic variables

In this correlation, leukocytes, neutrophils, platelets, blast cells, age, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels, the LDH level, and transfusion frequency were analyzed in patients with and patients without *HFE* gene variants. As assessed by Mann-Whitney-U-test, no significant differences were found in these correlations (data not shown). Patients with *HFE*-variants had a higher median ferritin level at diagnosis (409 µg/L) compared to patients without a detectable *HFE*-variant (346.5 µg/L) but this difference did not reach statistical significance (P=0.62) (**Figure 1A**).

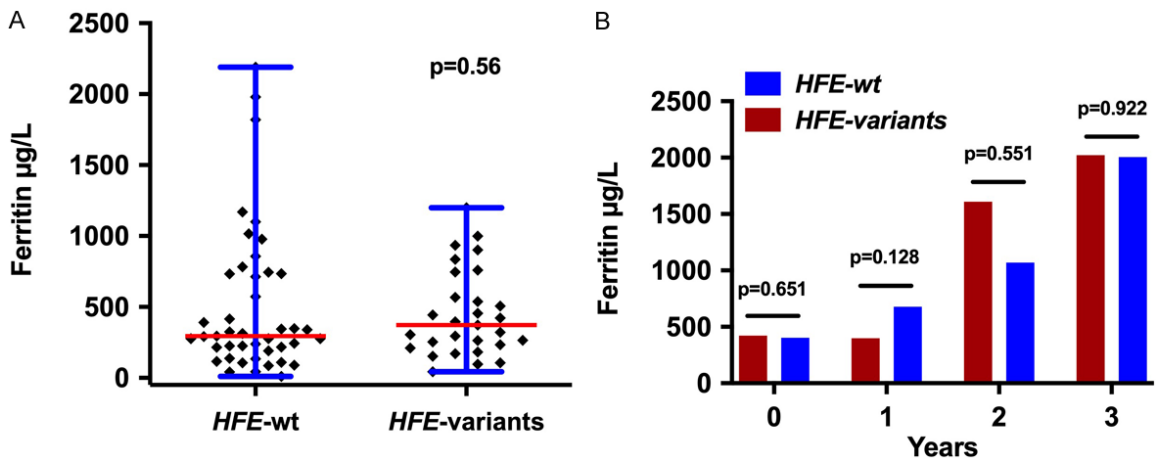

Figure 1. Effect of the *HFE* mutation status on the ferrtin levels during follow up. A: Median ferritin levels at diagnosis were slightly higher in patients with *HFE*-variants (409 µg/L; range: 23-7415) compared to *HFE* non-mutated patients (*HFE*-wt; 346.5 µg/L; range: 10-5450). B: During the first 2 years of transfusion therapy, patients with *HFE*-variants patients showed a slightly faster increase in serum ferritin levels compared to patients without *HFE*-variants (*HFE*-wt).

Increase in ferritin in MDS patients with or without HFE-variants over time

We next asked whether there is a faster increase in serum ferritin levels in transfused MDS patients harboring a *HFE*-variant compared to those without a *HFE*-variant, in this paper referred to as wild type *HFE* (*HFE*-wt). A rapid increase in serum ferritin levels was observed in patients with a *HFE*-variant and also in those with *HFE*-wt during the first three years of transfusion therapy (**Figure 1B**). However, there was a slightly faster increase in serum ferritin levels at 2 years observed in patients with *HFE*-variants (median ferritin level: 1610 µg/L) compared to MDS patients without a *HFE* mutation (*HFE*-wt) (median ferritin level: 1069 µg/L) (P=0.56).

Somatic mutations analyzed by NGS and correlation with HFE status

Somatic mutations are of increasing importance in patients with hematologic disorders, including MDS. To investigate a potential relationship between *HFE* gene variants and mutations in genes commonly affected in MDS, we performed a myeloid NGS gene panel analysis in 94 of our MDS patients. In 81 of these patients, at least one mutation was detected in neoplastic cells. However, no significant correlations were found when comparing *HFE*-variant expression with the most frequently detected somatic mutations in MDS (*ASXL1*, *SF3B1*, *SRSF2*, *TET2*, *DNMT3A*, *RUNX1*, *NRAS*, *TP53*, *IDH2* or *EZH2*) or when comparing the frequency of age-related somatic mutations, including CHIP mutations and more oncogenic mutations (CHOP) (**Figure 2A** and **2B**) [27].

Influence of HFE-variants on overall survival and progression-free survival in MDS

We also asked whether the *HFE*-variant status would serve as a prognostic parameter predicting the outcome in our patients. In particular, we asked whether overall survival (OS) or progression-free survival (PFS) is different when comparing MDS patients with *HFE*-variants with those carrying *HFE*-wt. In these analyses, we found a markedly better PFS in patients with *HFE*-variants compared to patients with *HFE*-wt (**Figure 3A**) (P=0.089). Within the first seven years of observation, patients with *HFE*-variants had a better OS compared to those carrying *HFE*-wt (**Figure 3B**) (P=0.44).

Discussion

MDS comprise a heterogeneous group of myeloid neoplasms characterized by clonal expansion of dysplastic myeloid cells, cytopenia(s), and an increased risk of progression to secondary AML [1-4]. Many patients suffer from chronic transfusion-dependent anemia, and several of them develop a significant iron overload [25,

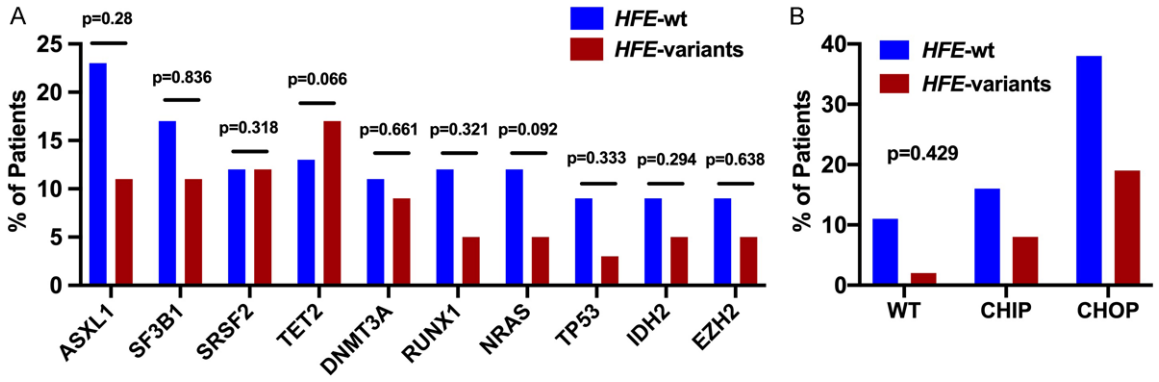

Figure 2. Somatic mutations analyzed by NGS according to *HFE* mutational status. A: A myeloid NGS gene panel was conducted in 94 of our MDS patients. In total 81 of all screened patients expressed at least on mutation. No significant correlations were found when comparing expression of *HFE* mutations with the most frequently detected somatic mutations in *ASXL1*, *SF3B1*, *SRSF2*, *TET2*, *DNMT3A*, *RUNX1*, *NRAS*, *TP53*, *IDH2* or *EZH2* detected by NGS. B: No significant correlation (assessed by Chi-square test) was found when comparing the frequency of mutations indicating clonal hematopoiesis of indeterminate potential (CHIP) or clonal hematopoiesis with oncogenic potential (CHOP) with the presence of *HFE*-variants in our MDS patients.

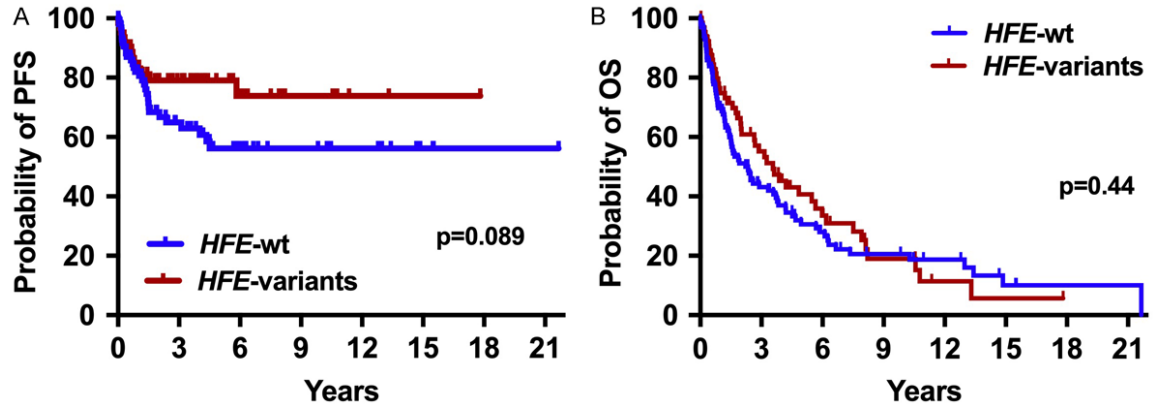

Figure 3. Progression-free survival and overall survival in MDS patients according to *HFE* mutation status. Progression-free survival (PFS) (A) and overall survival (OS) (B) was calculated according to the method of Kaplan and Meier. A clear but not significant difference in PFS between patients with *HFE*-variants (red line) and non-mutated patients (*HFE*-wt; blue line) was found (P=0.089 by log rank test). No differences in overall survival (OS) were found when comparing patients with *HFE*-variants (red line) with those without *HFE*-variants (*HFE*-wt; blue line) (P=0.44 by log rank test).

26, 32]. A key regulator of iron uptake and distribution is the *HFE* gene. The highlighting example of the impact of *HFE* is hereditary hemochromatosis, a genetic disorder characterized by massive iron overload. We analyzed the *HFE* status in patients with MDS and a group of healthy Austrian controls. We found a slightly higher prevalence of the *HFE*-variants H63D and C282Y in patients with low risk MDS compared to healthy Austrian controls. The median ferritin level at diagnosis was slightly higher in MDS patients with *HFE*-variants and the increase in serum ferritin in these patients within the first 2 years of transfusion therapy was faster compared to non-mutated (*HFE*-wt) patients. However, these differences were not statistically significant. Finally, we found that the frequency of *HFE* gene variants differ among MDS subgroups.

Only a limited number of studies have examined the frequency of *HFE*-variants in patients with MDS so far (Tables S1 and S2) [36-40, 47-50]. Looking at the patients' cohorts, the highest prevalence of MDS patients with *HFE* gene variants, namely 52%, was found in a small Hungarian study [36]. However, two other Hungarian studies reported a lower prevalence

of *HFE* gene mutations, namely 37.5% and 49.2% [37, 47]. This relatively large range in the prevalence of *HFE* gene variants may be explained by the small number of patients included in each of these studies. In our current study performed in Austria and in other similar studies performed in Croatia, the US, and Brazil, the prevalence of *HFE*-variants in MDS patients ranged between 30.8% and 39.2% [38, 40, 48]. A markedly lower prevalence of *HFE*-variants was found in MDS patients in Greece and China, with 27.8% and 4.1%, respectively [39, 50]. This variance in the prevalence can also be explained by the limited number of cases. Moreover, differences in the genetic background generated by ethnical or an environmental influence might be an alternative explanation for the varying prevalence of *HFE*-variants among these studies. Indeed, it is well known that the frequency of *HFE*-variants varies between different regions and areas (Table S3) [36, 37, 39, 40, 46-48]. Furthermore, the prevalence of particular *HFE*-variants, including C282Y and H63D, varies markedly among different populations, independent of the presence of MDS (Tables S1, S2, S3) [36-40, 45-50].

In our study, no significant difference in the prevalence of *HFE* gene variants between patients and healthy controls was observed. This is in line with data from the USA and Croatia [38, 48]. In contrast, the studies of Várkonyi et al. reported on an increased prevalence of *HFE* gene variants in patients with MDS compared to controls and similar data were published by de Souza et al. (Brazil) and Speletas et al. (Greece) [36, 37, 39, 40]. On the other hand, Nie et al. reported a lower prevalence of *HFE* gene variants in Chinese MDS patients [50]. Here, apart from the limited number of MDS patients included in all these studies, the heterogeneity of MDS could be an alternative or additional explanation for the different results obtained. This is greatly supported by the fact that the distribution of patients among the different FAB or WHO categories varies substantially in these studies.

The next question we asked was whether *HFE*-variants cluster in distinct MDS variants. A higher prevalence of *HFE*-variants was found in RA, RARS and CMML compared to RAEB and RAEB-T. However, we were not able to detect a significant difference among the various MDS subgroups defined by FAB or WHO criteria. This is in line with most studies published so far [48, 50]. Only Nearman et al. reported a significantly higher prevalence of *HFE* gene mutations in RARS patients (64.3%) [38]. A comparable but slightly lower prevalence of *HFE* gene mutations in RARS patients was found in our study (43.6%). These data are in contrast to the results reported from Croatian (17%) and Chinese (0%) study cohorts [38, 48-50]. Again, the limited numbers of patients with RARS included in these studies may explain the different results. In particular, there were only 11 and 12 patients with known RARS included in the Chinese and Croatian study, respectively, whereas the data of Nearman et al. (n=42) and our study (n=39) are based on higher numbers of RARS patients [38, 48, 50]. In this regard it is noteworthy to state, that similar to the US cohort the prevalence of *HFE*-variants was higher in our RARS patients compared to controls, although not statistically significant. Another interesting observation was that *HFE*-variant C282Y was not detectable in our patients with CMML whereas the *HFE*-variant H63D was detected in several CMML cases.

Despite the availability of novel potent chelators, transfusion-related iron overload and the resulting organ damage remain clinical challenges in the management of MDS patients. In some of these patients rapidly increasing levels of serum ferritin, a marker of iron-overload, are found. In our patients, relatively high ferritin levels were even detected at diagnosis before transfusion therapy started. Little is known about genetic and other factors predisposing for such a rapid iron overload in chronically transfused MDS patients so far. We asked whether *HFE*-variants may be triggers of rapid iron overload in our MDS patients. We observed that the median ferritin levels measured at diagnosis and after 24 months were higher in patients harboring *HFE*-variants compared to non-mutated patients. However, this difference did not reach statistical significance. These data show that neither the basal (pre-treatment) ferritin levels nor the *HFE* gene status are reliable biomarkers predicting the rapidity of iron overload in transfusion-dependent patients with MDS. In other words, a rapid iron overload may well be found also in patients without any *HFE*-variant which may be explained by the many other molecules and mechanisms that contribute to iron uptake, storage and

overload [25, 26, 32, 33, 36-40, 47]. On the other hand, a very rapid increase in serum ferritin levels may be indicative of primary hemochromatosis, especially when detected in transfusion-independent MDS patients. Therefore, we recommend to examine the *HFE* gene status in these patients. However, based on our data we do not recommend a *HFE* test in all patients with MDS. In fact, in our study, we did not detect major differences in the levels of serum ferritin at diagnosis when comparing patients heterozygous for *HFE* mutations with those carrying wild type *HFE*. It is also worth noting that in our cohort, no MDS patient was found to be homozygous for the clinically relevant *HFE*-variant C282Y. We also asked whether *HFE*-variants are associated with prognostic factors and scores as well as the outcome in MDS. The rate of progression-free survival was higher in patients with *HFE*-variants compared to patients with *HFE*-wt. Within the first seven years of observation a slightly better OS was observed in patients with *HFE*-variants, but this difference was no longer visible after 7 years of observation. This may be explained by complications related to cytopenia including infection or bleedings. In this regard it is noteworthy, that comorbidities are frequently found in these patients and that they represent an important predictive factor for survival [21-23]. In a next step, we analyzed whether *HFE*-variants correlate with single prognostic variables. However, no correlation was found between *HFE*-variants and various disease-related laboratory variables.

Previous and more recent data suggest that the development of MDS and subsequently AML is a step-wise process triggered by acquisition of somatic mutations, with subsequent molecular diversification and clonal selection in stem cell compartments [27]. The genetic background may be an important factor contributing to the somatic evolution, course and progression in these neoplasms. However, little is known about gene variants that are specifically involved in the pathogenesis of the disease or in the acquisition of somatic mutations. Our data show that *HFE*-variants are rather frequently detectable in patients with MDS. The underlying mechanism of this 'gene variant clustering' remains unknown. In order to investigate potential effects of *HFE* gene variants on the occurrence of MDS-related somatic mutations, we performed NGS using a myeloid gene panel containing the most frequent mutations associated with MDS and AML, in 94 of our MDS patients. The majority (86.2%) of screened MDS patients were found to have at least one mutation detected by targeted NGS. However, no correlations between myeloid gene panel mutations and the *HFE* mutation status were found.

In summary, our data show that *HFE*-variants are frequently detected in patients with MDS. The presence of these mutations was found to correlate with IPSS and IPSS-R subsets of our MDS patients and with their survival in follow up studies. Furthermore, the serum ferritin levels increased more rapidly over time in transfused patients compared to non-transfused patients with MDS. Although clear differences were seen, the significance level was not reached in all these comparisons which is most probably due to the relatively small number of cases examined in each cohort. Therefore, more studies with more patients are required to finally elucidate the role of *HFE*-variants in MDS.

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Disclosure of conflict of interest

P.V. has the following study-specific COI: Consultancy honoraria: Celgene, Novartis, Pfizer. In addition, P.V. has the following study-unrelated COI: Research grant: Pfizer, Celgene; consultancy honoraria: Blueprint, Incyte, Accord, Teva, Abbvie. W.R.S. has the following study-unrelated COI: honoraria from Novartis, Pfizer, AbbVie, Daiichi Sankyo, Amgen, Thermo Fisher, Deciphera, Incyte, Celgene and Jazz. G.H. has the following study-unrelated COI: honoraria from Novartis. K.V.G. has the following study-unrelated COI: honoraria from Novartis, Pfizer and Incyte.

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Table S1. Distribution of *HFE*-variants in MDS patients reported in various international studies

Study	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y*	H63D**	S65C	Heterozygous***	all variants
Present Study	8/167 (4.8%)	50/167 (29.9%)	3/167 (1.8%)	4/167 (2.4%)	65/167 (38.9%)
Varkonyi et al. (2003) [1]	4/50 (8%)	21/50 (42%)	n.t.	1/50 (2%)	26/50 (52%)
Varkonyi et al. (2006) [2]	5/61 (8.2%)	24/61 (39.3%)	n.t.	1/61 (1.6%)	30/61 (49.2%)
Varkonyi et al. (2006) [3]	2/32 (6.3%)	9/32 (28.1%)	n.t.	1/32 (3.1%)	12/31 (37.5%)
Lucijanic et al. (2016) [4]	2/36 (5.6%)	8/36 (22.2%)	n.t.	1/36 (2.8%)	11/26 (31%)
Speletas et al. (2003) [5]	0/54 (0%)	15/54 (27.8%)	n.t.	0/54 (0%)	15/54 (27.8%)
Nearman et al. (2007) [6]	15/148 (10.1%)	40/148 (27%)	n.t.	3/148 (2%)	58/148 (39.2%)
List et al. (2012) [7]	9/94 (9.6%)	24/94 (25.5%)	2/94 (2.1%)	0/94 (0%)	35/94 (37.2%)
De Souza et al. (2014) [8]	0/78 (0%)	23/78 (29.5%)	0/78 (0%)	1/78 (1.3%)	24/78 (30.8%)
Nie et al. (2010) [9]	0/271 (0%)	11/271 (4.1%)	n.t.	0/271 (0%)	11/271 (4.1%)

*Both homozygous and heterozygous for *HFE*-variant C282Y. **Both homozygous and heterozygous for *HFE*-variant H63D. ***Heterozygous for *HFE*-variants C282Y and H63D Abbreviations: MDS, Myelodysplastic Syndrome; n.t., not tested.

Table S2. Distribution of *HFE*-variants in healthy controls reported in various international studies

Study	*HFE*-variant, subjects (n) in subgroup (%)				
	C282Y*	H63D**	S65C	Heterozygous***	all variants
Present Study	42/494 (8.5%)	16/494 (24.7%)	n.d.	6/494 (1.2%)	164/494 (34.4%)
Varkonyi et al. (2003) [1]	4/80 (5%)	21/80 (26.3%)	n.t.	0/80 (0%)	25/80 (31.3%)
Varkonyi et al. (2006) [2]	12/171 (7%)	36/171 (21.1%)	n.t.	3/171 (1.8%)	51/171 (29.8%)
Varkonyi et al. (2006) [3]	n.t.	n.t.	n.t.	n.t.	n.t.
Lucijanic et al. (2016) [4]	11/200 (5.5%)	51/200 (25.5%)	7/200 (3.5%)	2/200 (1%)	71/200 (35.5%)
Speletas et al. (2003) [5]	0/264 (0%)	45/264 (17%)	n.t.	0/264 (0%)	45/264 (17%)
Nearman et al. (2007) [6]	204/2016 (10.1%)	525/2016 (26%)	n.t.	47/2016 (2.3%)	776/2016 (38.5%)
List et al. (2012) [7]	n.t.	n.t.	n.t.	n.t.	n.t.
De Souza et al. (2014) [8]	0/87 (0%)	5/87 (5.7%)	0/87 (0%)	0/87 (0%)	5/87 (5.7%)
Nie et al. (2010) [9]	0/1615 (0%)	169/1615 (10.5%)	n.t.	0/1615 (0%)	169/1615 (10.5%)

*Both homozygous and heterozygous for *HFE*-variant C282Y. **Both homozygous and heterozygous for *HFE*-variant H63D. ***Heterozygous for *HFE*-variants C282Y and H63D. Abbreviations: n.t., not tested.

Table S3. Distribution of *HFE*-variants in the general population in various countries

Country	Nr. of Subjects	*HFE*-variant frequency (%)			
		C282Y*	H63D**	Heterozygous***	all variants
Europe					
Austria (present study)	494	8.5%	23.5%	1.2%	33.3%
Hungary [1]	80	5%	26.3%	0%	31.3%
Hungary [2]	171	7%	21.1%	1.8%	29.8%
Croatia [4]	200	5.5%	25.5%	1%	35.5%⁺
Greece [5]	264	0%	17%	0%	17%
Scandinavia [10]	837	8.6%	15.7%	0.8%	25.1%
France [11]	410	12.7%	25.1%	2.2%	40%
Germany [12]	500	7.4%	19.2%	1.8%	28.4%
Russia [13]	840	6.7%	27.5%	1.2%	35.4%
Slovenia [14]	1282	7.1%	23.2%	0.9%ˣ	34.9%°
North Italy [15]	1132	5.1%	23.5%	1.4%ˣ	32%•
Denmark [16]	2501	9.6%	23.5%	1.4%	34.5%
North America					
American [6]	2016	10.1%	26%	2.3%	38.5%
American Caucasian [17]	44082	10.6%	26.3%	2.1%	39%
American Indian [18]	645	5.7%	20.9%	1.1%	27.7%
Mexico [19]	153	2%	12.4%	0.7%	15.1%
South America					
Brazil [8]	87	0%	5.7%	0%	5.7%
Africa					
Tunisia [20]	570	0%	30.4%	0%	30.4%
North Africa [21]	342	0.9%	13.2%	n.t.	14.1%
South Africa [22]	236	0%	0.4%	0%	0.4%
Western Africa [23]	286	0%	2.5%	0%	2.5%
Oceania					
Australia [24]	3375	11.8%	24.2%	2%	38%
Asia					
China [9]	1615	0%	10.5%	0%	10.5%
China [25]	395	0%	4.6%	0%	4.6%
Japan [26]	252	0%	2%	0%	2%
Korea [27]	484	0%	8.5%	0%	8.5%
North India [28]	100	0%	11%	0%	11%
Thailand [29]	380	0%	5.6%	0%	5.6%

*Both homozygous and heterozygous for *HFE*-variant C282Y. **Both homozygous and heterozygous for *HFE*-variant H63D. ***Heterozygous for *HFE*-variants C282Y and H63D. ⁺*HFE*-variant S65C in 3.5% of tested healthy controls. ˣHeterozygous for either H63D/S65C, H63D/C282Y and S65C/C282Y. °*HFE*-variant S65C in 3.7% of tested healthy controls. •*HFE*-variant S65C in 1.9% of tested healthy controls. Abbreviations: n.t., not tested, Nr., number.

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