Cytokine and Gene Expression Profiling in Patients with HFE-Associated Hereditary Hemochromatosis according to Genetic Profile

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Abstract

Background: Hemochromatosis gene (HFE)-associated hereditary hemochromatosis (HH) is characterized by down-regulation of hepcidin synthesis, leading to increased intestinal iron absorption. Objectives: The objectives were to characterize and elucidate a possible association between gene expression profile, hepcidin levels, disease severity, and markers of inflammation in HFE-associated HH patients. Methods: Thirty-nine HFE-associated HH patients were recruited and assigned to 2 groups according to genetic profile: C282Y homozygotes in 1 group and patients with H63D, as homozygote or in combination with C282Y, in the other group. Eleven healthy first-time blood donors were recruited as controls. Gene expression was characterized from peripheral blood cells, and inflammatory cytokines and hepcidin-25 isoform were quantified in serum. Biochemical disease characteristics were recorded. Results: Elevated levels of interleukin 8 were observed in a significant higher proportion of patients than controls. In addition, compared to controls, gene expression of ζ-globin was significantly increased among C282Y homozygote patients, while gene expression of matrix metalloproteinase 8, and other neutrophil-secreted proteins, was significantly upregulated in patients with H63D. Conclusion: Different disease signatures may characterize HH patients according to their HFE genetic profile. Studies on larger populations, including analyses at protein level, are necessary to confirm these findings.

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Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disorder caused by mutations in genes involved in iron metabolism, which results in increased intestinal iron absorption. The clinical manifestations are related to excessive iron accumulation in parenchymal cells of vital organs such as the liver, heart, and endocrine glands in particular. Thus, patients may develop liver disease, arthralgia/arthritis, diabetes mellitus, impotence, skin pigmentation, and heart enlargement.

HH is most commonly associated with mutations in the hemochromatosis gene (HFE) [1–5]. The HFE gene

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encodes the HFE protein, a nonclassical major histocompatibility type Ib membrane protein, which interacts with the transferrin receptors Tfr1 and Tfr2 at the cell surface. This interaction controls normal expression of hepcidin, a key hormone regulating iron homeostasis [3, 5]. HFE is expressed in a wide range of tissues and cells, including Kupffer and epithelial cells of the liver, enterocytes of the duodenum, and gastric epithelial cells, as well as macrophages, circulating monocytes, and granulocytes. Conversely, HFE has been shown to localize inside the intracellular granules in granulocytes [6]. Different splice variants of the HFE gene have been identified in different tissues, expressed at different levels, and include a soluble variant produced in the duodenum, lacking its transmembrane domain and cytoplasmic tail [7]. The different splice variants might reflect different functions of HFE in the different tissues. In addition to its role in iron metabolism, HFE has recently emerged as a potential key molecule in the immunological response, bridging the innate and adaptive immune responses [8].

More than 80% of HH patients with mutations in HFE are homozygote for the C282Y mutation that impairs correct folding and posttranslational processing of HFE needed for its cell surface expression [9]. An additional 14% of HH patients harbor a H63D mutation, either as heterozygote with C282Y or as homozygote [1–3, 10]. The H63D mutation does not alter the cell surface expression of HFE but rather alters its affinity for Tfr1 [11]. Those patients tend to have a lower degree of iron load compared with C282Y homozygotes [3, 12]. For other genotypes, a small enhancement in iron absorption may occur, but this rarely results in clinically relevant iron modification and development of HH [1, 3]. Heterozygous HFE mutation is common in Caucasian populations. The overall allele frequency for C282Y mutation is 7–8% in the Norwegian population [13–16].

The initial diagnostic approach is by finding elevated transferrin saturation (TfSat) and an increased serum or plasma ferritin above the upper limits of normal without the presence of significant inflammation or infection. Different studies have used different TfSat cutoff values, but TfSat ≥45% has high sensitivity for detecting C282Y homozygotes. Current treatment guidelines for HH patients are phlebotomy (venesection) and dietary advice [17]. Phlebotomy is performed when serum ferritin (SF) levels exceed 300–1,000 µg/L or when patients are symptomatic.

Hepcidin is produced in the liver and is a key regulator of systemic iron homeostasis [1, 5, 10]. The synthesis of hepcidin is normally upregulated by high iron blood levels, inflammation, or as a response to infection. Hepcidin binds to the cellular iron exporter ferroportin (FPN) located on the basolateral membrane of hepatocytes and enterocytes and in macrophages and thereby restrains iron from entering the blood circulation [1, 5, 10]. Hepcidin exists in several isoforms (hepcidin-25, -24, -23, -22, -20, and -19) [18], but hepcidin-25 (Hep25) has the highest affinity for FPN, and it is this isoform that has a central role in iron regulation [19]. Hep25 synthesis in HH patients is downregulated causing excess FPN-mediated iron export and increased dietary iron uptake from enterocytes in the intestine and iron export from macrophages. Consequently, increased iron levels in the blood stream are observed [1, 4, 5]. The altered immunological status observed in HFE-associated HH patients is mainly due to iron overload, leading, for example, to lower levels of circulating invariant natural killer cells [20] or lower levels of cytokine production in macrophages [21]. In C282Y HH patients, the lower levels of expression of MHC class I molecules shown in peripheral mononuclear cells have been attributed to impaired intracellular trafficking due to HFE failing to bind to β2-microglobulin [22, 23]. A recent in vitro study evidenced a difference between wild-type and C282Y HFE proteins in their ability to activate CD8+ T lymphocytes, suggesting that C282Y mutation may confer a stronger cellular immunity response in individuals harboring it [24]. On the other hand, autoimmune conditions are common among HH patients [25].

Gene expression profiling has proved useful in identifying unique signatures in a range of diseases [26–29]. Given the ubiquitous expression of HFE and its potential role in immunological processes, one main aim of the present study was to analyze gene expression from peripheral blood cells of HFE-associated HH patients according to their genetic profile, in order to identify novel molecular characteristics of the disease that may prove useful as biomarkers. The other aim was to characterize inflammatory cytokine profiles, hepcidin levels, and iron status in the patient population, to elucidate possible associations between these parameters.

Materials and Methods

Study Population and Ethics

Samples from 39 consecutive hemochromatosis patients, with known HFE mutation status and confirmed high SF levels, were collected. Information on whether the patients had phlebotomy treatment within the last 6 months prior to study inclusion was recorded. Patients who were C282Y homozygotes were assigned to...
patient group 1 and those who were H63D homozygotes or H63D/C282Y compound heterozygotes were assigned to patient group 2. Control samples were collected from 11 healthy newly recruited blood donors, who had never given blood prior to their enrollment in the study. Study participants in the control group were selected with regard to sex and age to match as much as possible the patient groups.

Sample Collection

Peripheral venous blood samples were collected according to the standard operating procedures at Østfold Hospital Trust, Norway. Serum samples were collected in Vacuette® tubes 9 mL Z Serum Separator Clot Activator (Greiner Bio-One, Kremsmünster, Austria) and allowed to clot at room temperature for 1 h prior to centrifugation at 2,100 g for 10 min. Serum was removed and stored at −80°C until use. Samples used for miRNA and microarray analysis were collected in PAXgene Blood RNA Tubes (Qiagen, Venlo, the Netherlands), frozen stepwise, according to the manufacturer’s instruction, and stored at −80°C until use. Whole blood samples were collected in Vacuette® tubes 9 mL K2 E K2EDTA (Greiner Bio-One) and stored at room temperature for maximum 1 h prior to analysis.

Hematological Analysis and Liver Enzymes

Hematological parameters including hemoglobin, erythrocytes, erythrocyte volume fraction, mean corpuscular volume, and mean corpuscular hemoglobin, reticulocyte particle concentration, mean corpuscular hemoglobin, reticulocyte hemoglobin content, and reticulocyte count were analyzed within 1 h after obtaining the samples on a CELL-DYN Sapphire hematology analyzer (Abbott, Chicago, IL, USA). Iron status parameters including serum iron, ferritin, transferrin, and total iron-binding capacity and liver enzymes including alanine aminotransferase, gamma-glutamyl transferase, and alkaline phosphatase were measured on ADVIA Chemistry System (Siemens, München, Germany). TSat was calculated from total iron-binding capacity and iron serum values. Soluble transferrin receptors were analyzed in thawed serum samples using UniCel DxI 800 (Beckman Coulter, Brea, CA, USA).

Hepcidin Isolation and Quantification

Hep25 was analyzed on 49 samples from patients and control, as one sample in group 2 was not available for analysis. Isotope-labeled ([13C9,15N]Phe4,9) Hep25 (Bachem, Bubendorf, Switzerland) was used as internal standard (100 ng/mL in aqueous 0.1% formic acid), where 100-µL internal standard solution was added to 200-µL serum. Hepcidin was isolated by solid phase extraction as described [30], except 2 x 80 µL acetonitrile-water-acetic acid (50:50:2, v/v) was used for elution. The extracts were analyzed by liquid chromatography high-resolution mass spectrometry on an Accela open autoinjector, Accela 1250 pump, and a Q Exactive high-resolution mass spectrometer (Thermo Scientific, Waltham, MA, USA). Separation was conducted on a Polaris C18A (50 x 2 mm; Agilent) reversed-phase chromatography column, using a gradient from 20% acetonitrile in 0.1% formic acid in water (t = 0 min) linearly increased to 70% acetonitrile over 5 min. Hep25 was quantified by extracting the mass traces of the first 4 isotopomers of [M + 4H]4+ (i.e., m/z 697.7637, 698.0144, 698.2644, and 698.5145) with a width of 8 ppm. Hep25 spiked at 2, 8, 32, 64, and 256 ng/mL in rabbit serum was used as calibrators.

Inflammation Cytokines and Chemokines

Twelve inflammatory cytokines and chemokines (IL1A, IL1B, IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFNy, TNFα, and GM-CSF) were profiled in serum using Multi-Analyte ELISArray Kit (Qia-gen). The detection was performed using Epoch™ Microplate Spectrophotometer (BioTek, Winooski, VT, USA) and analyzed using the Gen5 software (BioTek). Optical density values obtained in ELISA were used to detect whether elevated levels of the different cytokines were observed in serum and to compare levels of cytokines in serum with other parameters in the study.

RNA Extraction from Whole Blood

Total RNA from whole blood stabilized in PAXgene Blood RNA Tubes was isolated and purified using the PAXgene Blood miRNA kit (Qiagen) and stored at −80°C until use. RNA integrity was confirmed using Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray

Gene expression profiling was performed on 12 randomly selected samples in group 1 and 6 of each group 2 and the control group. GeneChip WT PLUS Reagent Kit was used on total RNA (150 ng) as described by the manufacturer’s instructions for whole genome gene expression analysis (Affymetrix, Santa Clara, CA, USA). Labeled and fragmented single-stranded cDNAs were hybridized to GeneChip Human Gene 2.0 ST Arrays (Affymetrix) covering 40,716 RefSeq transcripts. The arrays were washed and stained using the FS-450 fluids station (Affymetrix). Signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, USA). The scanned images were processed using AGCC (Affymetrix GeneChip Command Console) software (Affymetrix).

Further bioinformatics analysis was conducted on the transcripts considered significant in the statistical analysis (p < 0.05 and FC >1.2) to identify functional significance by means of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA). In brief, the data set containing gene identifiers and corresponding fold changes and p values was uploaded into the web-delivered application, and each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. The functional analysis identified the biological functions and/or diseases that were most significant to the data sets. Fisher’s exact test was performed to calculate the p value determining the probability that each biological function and/or disease assigned to the data set was due to chance alone. The data sets were mined for significant pathways with the IPA library of canonical pathways, and networks were generated by using IPA as graphical representations of the molecular relationships between genes and gene products.

cDNA Synthesis and qPCR

Ten µl of total RNAs were converted to single-stranded cDNA by using High-Capacity cDNA Reverse Transcription Master Mix as instructed (Applied Biosystems, Foster City, CA, USA). Individual TaqMan Gene Expression Assays (Applied Biosystems) were performed for the following genes: human ζ-globin, (HBZ; Assay ID: Hs00923579_m1), lactotransferrin (LTF; Assay ID: Hs00914334_m1), matrix metallopeptidase 8 (MMP8; Assay ID: Hs01029057_m1), α-defensin (DEFA1, DEFA3, and DEFA1B; As-
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Data Analysis and Statistics

Descriptive analyses and frequencies were employed to assess sample characteristics. Data distributions were tested for normality in all cases. Comparisons of blood markers of iron status, hematological parameters, inflammation, liver enzymes, and mRNA profiles were performed using one-way analyses of variance (ANOVA) followed by Tukey’s post hoc test. To account for non-normality, comparisons were assessed using the Kruskal-Wallis test followed by Mann-Whitney U tests. Fisher’s exact test was used to compare proportions. Independent sample t test or, to account for non-normality, Mann-Whitney U test was performed for comparison of binary variables. To assess possible associations between variables, Pearson’s correlation (r) or, to account for non-normality, Spearman’s product-moment correlation (rho) was used. A p value of <0.05 was defined as statistically significant. All the analyses of blood markers were performed using SPSS 23 for Windows (IBM, Armonk, NY, USA).

For gene expression analysis, the Affymetrix CEL files (containing probe intensities) were imported into Partek Genomics Suite software (Partek Inc., Chesterfield, MO, USA) for statistical analysis. Robust microarray analysis yielding normalized log2-transformed signal intensities was applied for normalization. Gene transcripts with maximal signal values of <5 (log2 values) transformed signal intensities was applied for normalization. One-way ANOVA as implemented in Partek Genomics Suite software (Partek Inc., Chesterfield, MO, USA) for statistical analysis. Robust microarray analysis yielding normalized log2-transformed signal intensities was applied for normalization. Genes with relative gene expression could be observed in association with phlebotomy in 6 months prior to study enrollment and dividing patients between those who did receive phlebotomy or not detectable Hep25 in their serum. To elucidate if a small amount of Hep25 could be associated with any of the disease severity parameters in this study, statistical tests were conducted to compare these parameters for the dichotomous variable, detectable Hep25 or not detectable Hep25 within the patients. Ferritin was the only variable displaying significant higher values for patients with detectable Hep25 (median 198, IQR = 291, n = 17) compared to patients with no detectable Hep25 (median = 86, IQR = 282, n = 21; U = 96, z = 2.42, p = 0.015, r = 0.44). When further dividing patients between those who did receive phlebotomy the last 6 months prior to study enrollment and those who did not, no significant difference in Hep25 could be observed in association with phlebotomized status (p = 0.674).

Cytokine Profiles

Several cytokines were analyzed (IL1α, IL1β, IL2, IL4, IL6, IL8, IL10, IL12, IL17, IFNγ, TNFα, and Gm-CSF) in order to investigate the inflammatory status of the study population. As opposed to none of the individuals in the control group, 42% and 46% of the patients in groups 1 and 2, respectively, had elevated levels of IL8 in serum (χ² = 7.32, df = 2, p = 0.026). No significant differences between the patients and control group were observed for any other of the cytokines (Table 2).
Several patients among those who underwent phlebotomy within the last 6 months had elevated IL8 level \((n = 13/21)\) compared to patients who were not recently phlebotomized \((n = 4/12)\) \((\chi^2 = 4.98, df = 1, p = 0.045)\). We observed no other statistical differences in disease parameters in this study between patients with elevated levels of IL8 in serum and the patients with no IL8 in serum.

### Table 1. Characteristics of demographics and blood markers of iron status, hematological parameters, and liver enzymes in HH patient groups (1–2) compared to the control group (3)

| Characteristics          | Subjects with HH diagnosis | Subjects with no HH diagnosis |
|--------------------------|-----------------------------|------------------------------|
|                          | Patient group 1 | Patient group 2 | Control group |
| **HFE genotype**         | C282Y/C282Y | H63D/H63D or H63D/C282Y | Control |
| N                        | 26            | 13               | 11               |
| Gender (male/female)     | 15/11         | 12/1             | 7/4              |
| Age, years               | 52.5±10.4     | 58.2±9.3         | 45.9±5.3         |
| Recent phlebotomy (yes/no) | 13/11         | 8/5              |                 |

**Iron status**

| Hepcidin, nM              | 0.7±0.9*** \((n = 25)\) | 0.6±0.8*** \((n = 5)\) | 10.5±4.8       |
| Detectable level          | 1.4±0.8 \((n = 12)\)     | 1.6±0.4 \((n = 5)\)    |                 |
| No detectable level       | 0 \((n = 13)\)            | 0 \((n = 8)\)           |                 |
| SF, µg/L\(^c\)            | 171 (258)                | 109 (172)              | 104 (98)        |
| Nonelevated SF            | 89 (94) \((n = 14)\)     | 86 (116) \((n = 11)\)  |                 |
| Elevated SF               | 394 (174) \((n = 12)\)   | 657 (596) \((n = 2)\)  |                 |
| TIBC, µmol/L              | 50.9±7.4***, ^^^         | 65±5 \((n = 12)\)      | 65±12           |
| Serum iron, µmol/L        | 32.8±8.1***             | 26.3±12.1** \((n = 12)\) | 15.3±4.5   |
| TfSat, %                  | 66.4±20.2***, ^^^         | 41.7±18.0 \((n = 12)\) | 23.9±7.9   |
| TfR, mg/L                 | 0.87±0.22 \((n = 19)\)   | 0.98±0.26 \((n = 9)\)  | 1.01±0.18 \((n = 6)\) |

**Hematological parameters**

| Hemoglobin, g/dL          | 15.7±1.1       | 16.0±1.1       | 15.3±1.3       |
| EVF                      | 0.46±0.3       | 0.47±0.3       | 0.46±0.03      |
| EPC, \(×10^{12}/L\)      | 4.7±0.3        | 4.9±0.3        | 5.0±0.4        |
| MCV, fL                  | 97.5±4.0***    | 95.5±3.6       | 92.3±2.6       |
| MCH, pg                  | 33.4±1.3***    | 32.6±1.0***    | 30.8±0.9       |
| CHr, pg                  | 34.1±1.4***    | 33.8±1.3***    | 32.1±0.7       |
| Retic, \(×10^9/L\)       | 79.6±28.3      | 68.9±21.8 \((n = 11)\) | 62.6±14.6   |

**Liver enzymes**

| GT, U/L                  | 32.6±20.1      | 42.1±18.5      | 27.7±11.1      |
| ALAT, U/L                | 31.6±15.6      | 39.0±18.9      | 40.2±25.8      |
| ALP, U/L                 | 76.1±19.7      | 67.5±25.3      | 72.8±15.0      |

Data are presented as mean ± SD, unless indicated otherwise. HH, hereditary hemochromatosis; HFE, hemochromatosis gene; SF, serum ferritin; TIBC, total iron-binding capacity; TfSat, transferrin saturation; TfR, transferrin receptor; EVF, erythrocyte volume fraction (hematocrit); EPC, erythrocyte particle concentration; MCV, mean corpuscular volume erythrocytes; MCH, mean corpuscular hemoglobin erythrocytes; CHr, reticulocyte hemoglobin content; Retic, reticulocyte count; GT, gamma-glutamyl transferase; ALAT, alanine aminotransferase; ALP, alkaline phosphatase. \(^a\) C282Y, cDNA, nucleotide 845 G→A; H63D, cDNA, nucleotide 187 C→G. \(^b\) Two individuals were blood donors, not included. \(^c\) Presented as median (interquartile range). \(^d\) Elevated SF is defined as >300 and >200 µg/L in men and women, respectively. ** \(p < 0.01\), *** \(p < 0.001\) compared to control; ^^^ \(p < 0.001\) compared to the HH group.

#### Gene Expression Profiles and qPCR Analysis

Gene expression data set for the 24 randomly selected study samples is available from the Gene Expression Omnibus (GEO) database (Accession No. GSE121620). Out of the 18,857 transcripts analyzed, 19 genes were found overall to be ≥2-fold up- or downregulated in HH patient groups compared to healthy controls, according to their genetic profile, and 9 genes were differentially expressed...
in C282Y homozygotes, compared to H63D harboring patients (Table 3). When collectively comparing all samples from the 2 patient groups to healthy controls under IPA or when comparing each patient group to healthy controls, pathways related to inflammatory response, infectious disease, respiratory disease, hematological disease, dermatological diseases and condition, organismal injury and abnormalities, and cancer were identified (see online suppl. files 1–3; for all online suppl. material, see www.karger.com/doi/10.1159/000511551).

ζ-Globin (HBZ), α-defensin (DEFA1, DEFA1B, and DEFA3) hereafter named DEFA, lactotransferrin (LTF), matrix metallopeptidase 8 (MMP 8), and major histocompatibility complex, class II DR beta 1 (HLA-DRB1) and beta 5 (HLA-DRB5) genes were selected for further verification by qPCR on the study population (n = 50 for the first 4 genes and n = 45 for the HLA genes) based on profiling results and potential relevance to iron metabolism regulation and hemochromatosis. No fold changes were calculated for HLA-DRB5, as this gene was expressed in less than half of the samples overall. No significant difference in HLA-DRB1 between either of the patient groups and the control group could be confirmed by qPCR (Table 4). When further dividing patients in the C282Y homozygotous group between those who did receive phlebotomy the last 6 months prior to study enrollment (n = 11 for HLA-DRB1 and n = 12 for the other genes) and those who did not (n = 11 for HLA-DRB1 and n = 12 for the other genes), no significant differences between these subgroups were observed for any of the genes.

Statistical analysis of possible associations between upregulated gene expression and the disease parameters in this study revealed for HBZ no significant bivariate correlations within the C282Y homozygotous group. Within the patients harboring an H63D mutation, MMP8 levels correlated with levels of SF (rho [p] = 0.599, p = 0.031, n = 13), and there was a significantly lower level of LTF in patients with no detectable Hep25 in serum compared to patients with a small amount of Hep25 (t[11] = 2.404, p = 0.035, eta square = 0.34, n = 13).

### Table 2. Cross table of inflammatory cytokine status in serum in study population

| Cytokine | N  | Group 1a (+/−) | Group 2b (+/−) | Control (+/−) | Statistics (χ², df, p) |
|----------|----|----------------|----------------|---------------|-----------------------|
| IL1α     | 50 | 4/22           | 3/10           | 5/6           | χ² = 3.84, df = 2, p = 0.147 |
| IL1β     | 50 | 6/20           | 3/10           | 3/8           | χ² = 0.083, df = 2, p = 0.959 |
| IL2      | 50 | 5/21           | 4/9            | 6/5           | χ² = 4.59, df = 2, p = 0.010 |
| IL4      | 50 | 3/23           | 4/9            | 4/7           | χ² = 3.56, df = 2, p = 0.168 |
| IL6      | 50 | 3/23           | 1/12           | 1/10          | χ² = 0.155, df = 2, p = 0.925 |
| IL8      | 50 | 11/15          | 6/7            | 0/11          | χ² = 7.32, df = 2, p = 0.026 |
| IL10     | 50 | 6/20           | 4/9            | 2/9           | χ² = 0.543, df = 2, p = 0.762 |
| IL12     | 50 | 6/20           | 3/10           | 5/6           | χ² = 2.13, df = 2, p = 0.345 |
| IL17A    | 50 | 9/17           | 7/6            | 7/4           | χ² = 3.05, df = 2, p = 0.217 |
| IFNγ     | 50 | 7/19           | 5/8            | 6/5           | χ² = 2.60, df = 2, p = 0.272 |
| TNFa     | 50 | 5/21           | 2/11           | 2/9           | χ² = 0.087, df = 2, p = 0.957 |
| Gm-CSF   | 50 | 4/22           | 5/8            | 5/6           | χ² = 4.42, df = 2, p = 0.110 |

a HFE genotype C282Y/C282Y. b HFE genotype H63D/H63D or H63D/C282Y.
Discussion

In this study, we have characterized a patient population with HFE-associated HH according to their genetic profile. Blood markers of iron status, hematological parameters, liver enzymes, inflammation markers, Hep25, and total gene expression from peripheral blood cells were analyzed. We found significant differences in the gene expression of HBZ in patients homozygous for C282Y and in the gene expression of several neutrophil-associated proteins, in particular MMP8, in patients harboring a H63D mutation. IL8 was the only proinflamma-

| Patient group | Gene symbol | Gene assignment | Biological process | Fold change | p value |
|---------------|-------------|-----------------|--------------------|-------------|---------|
| 1             | **HLA-DRB5** | Major histocompatibility complex, class II, DR beta 5 | Immunoglobulin production, involved in immunoglobulin-mediated immune response | 18.7 | 0.037 |
|               | **HLA-DRB1** | Major histocompatibility complex, class II, DR beta 1 | Immunoglobulin production involved in immunoglobulin-mediated immune response | 13.4 | 0.035 |
|               | **HBZ**     | ζ-Globin (globin, zeta) | Negative regulation of transcription from RNA polymerase II promoter, oxygen transport, erythrocyte maturation | 3.6 | 0.012 |
|               | **DEFA3**   | α-Defensin 3, neutrophil specific | Innate immune response in mucosa, antimicrobial humoral response | 2.3 | 0.022 |
|               | **MT-TD**   | Mitochondrially encoded tRNA aspartic acid | – | 2.2 | 0.010 |
|               | **CEACAM8** | Carcinoembryonic antigen-related cell adhesion molecule 8 | Neutrophil degranulation, immune response, leukocyte migration | 2.1 | 0.012 |
|               | **DEFAB1**  | α-Defensin 1B | Innate immune response in mucosa, antiviral defense | 2.1 | 0.038 |
|               | **CFD**     | Complement factor D (adipsin) | Complement activation, proteolysis, neutrophil degranulation | −2.3 | 0.004 |
| 2             | **IGHJ6**   | Immunoglobulin heavy joining 6 | – | 7.5 | 0.043 |
|               | **CEACAM8** | Carcinoembryonic antigen-related cell adhesion molecule 8 | Neutrophil degranulation, immune response | 5.1 | 0.008 |
|               | **LTF**     | Lactotransferrin | Humoral immune response, DNA-templated transcription, neutrophil degranulation, regulation of cytokine production | 3.9 | 0.014 |
|               | **MMP8**    | Matrix metallopeptidase 8 (neutrophil collagenase) | Extracellular matrix disassembly, proteolysis, neutrophil degranulation | 2.8 | 0.038 |
|               | **OLEM4**   | Olfactomedin 4 | Cell adhesion, neutrophil degranulation | 2.8 | 0.005 |
|               | **CEACAM6** | Carcinoembryonic antigen-related cell adhesion molecule 6 | Signal transduction, neutrophil degranulation | 2.8 | 0.041 |
|               | **RNU6-949P** | RNA, U6 small nuclear 949, pseudogene | – | 2.3 | 0.029 |
|               | **RNU7-22P** | RNA, U7 small nuclear 22 pseudogene | – | 2.0 | >0.001 |
|               | **CRISP3**  | Cysteine-rich secretory protein 3 | Neutrophil degranulation, defense response | 2.0 | 0.004 |
|               | **GBP 1**   | Guanulate-binding protein 1, interferon inducible | Negative regulation of: interleukin-2 secretion, T-cell receptor signaling, and ERK1 and ERK2 cascade. Interferon-gamma-mediated signaling pathway. | 2.0 | 0.024 |
|               | **ZNF208**  | Zinc finger protein 208 | Regulation of transcription | 2.0 | 0.014 |

HH, hereditary hemochromatosis. * Patient group 1, C282Y homozygotes; patient group 2, H63D homozygotes plus H63D/C282Y heterozygotes. Healthy subjects were assigned to the control group.
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The results from blood markers of iron, hematological, and liver status support previous findings for HH patients and show that the study population is representative for this patient group [33–35]. One of the main findings reported herein is the overexpression of HBZ among patients with the C282Y HFE mutation. Hemoglobin is composed of 2 α-like and 2 β-like globin chains expressed in balanced amounts in normal erythroid cells. The 2 separated α-like and β-like globin gene clusters are subject to several levels of regulation that ensure assembly of embryonic (ζ2ε2), fetal (α2γ2), and adult (α2β2) hemoglobins during development [36,37], where embryonic and fetal hemoglobins have higher affinity for oxygen than adult hemoglobin. In particular, ζ-globin has a very high affinity for oxygen, allowing the embryo to capture oxygen from maternal hemoglobin through the placenta. However, delivery of oxygen to tissue is much lower with ζ-globin than with α-globin, and the switch from ζ- to α-globin occurs through silencing within the first 10 weeks of gestation [38–40].

Nevertheless, we found that HBZ was upregulated in HH patients homozygote for C282Y with >3-fold compared to controls. Our observation is not the first report of ζ-globin being expressed in detectable levels in adult tissue, as detection of ζ-globin in subjects suffering from α-thalassemia has previously been reported [41–43]. In addition, the presence of embryonic globin mRNAs has been reported in small amounts in adult erythroblasts and reticulocytes [44,45].

Whether the upregulation of ζ-globin mRNA expression in C282Y HH patients is a consequence of the disease or a physiological attempt to compensate for its detrimental effects can only be speculated on at this point. The genes in the α-globin locus are surrounded by a cluster of transcriptionally active ubiquitously expressed genes in the subtelomeric region of chromosome 16 and are expressed through histone hyperacetylation and remodeling of the local chromatin structure in erythroid cells. The major α-like globin gene transcriptional regulatory element, the DNase hypersensitive site HS40, is located in an intron of the adjacent housekeeping gene nitrogen permease-regulator-like gene 3 (NPRL3) [46–48]. Interestingly, data from our microarray gene expression profiling identified NPRL3 as being significantly upregulated by nearly 50% (fold change 1.49, \( p = 0.02 \)) in C282Y HH patients compared to controls, while no upregulation of this gene was observed in H63D mutation-carrying patients. The interaction between the expression of NPRL3 and both overall expression of α-like globin genes and the possible reactivation of mRNA expression of ζ-globin, that is located immediately downstream of NPRL3, remain to be elucidated. Further investigations at the protein level are required to identify whether a hypothetically semiembryonic hemoglobin such as Portland-2 (ζβ2), a protein believed to be compatible with normal adult physiology [49], is produced in C282Y homozygous HH patients and to unveil potential physiological effects on oxygen delivery to tissues and iron-catalyzed oxidative cellular damage. As no upregulation of expression of this gene was observed in HH patients with H63D even though most of them were compound heterozygous C282Y/H63D, increase in ζ-globin mRNA production is likely linked to a total impairment of cell surface localization of the HFE protein.

| Gene symbol | Gene assignment | Patient group 1 | Patient group 2 |
|-------------|-----------------|-----------------|-----------------|
| HBZ         | ζ-Globin        | 3.3**           | 1.2             |
| DRB1        | HLA class II, DR beta 1 | −1.2          | −2.2            |
| MMP8        | Matrix metallopeptidase 8 | 1.5        | 3.2**           |
| DEFA1+DEFA1B+DEFA3 | α-Defensin | 1.8           | 2.9*             |
| LTF         | Lactotransferrin | 1.7           | 2.3*             |

HH, hereditary hemochromatosis. Patient group 1, C282Y homozygotes; patient group 2, H63D homozygotes plus H63D/C282Y heterozygotes. Healthy subjects were assigned to the control group for fold change analyses. * \( p < 0.05 \), ** \( p < 0.01 \) compared to control.
of increased MMP8 transcription that correlated with SF
cytokine as a biomarker of disease severity. ζ-Globin
gene expression was highly upregulated in C282Y
homozygotes but not in H63D HH patients or H63D/ C282Y, suggesting its association with hemochromato-
sis where cellular membrane expression of HFE is com-
pletely abolished. In addition, gene expression of MMP8
and other neutrophil-associated proteins was upregu-
lated in HH patients harboring the H63D mutation. Our
results suggest that different disease signatures are in-
volved in HH patients depending on the HFE genetic
profile. However, studies on a larger population, includ-
ing analysis at protein level, are necessary to confirm our
findings.

Conclusion

In this study, we identified IL8 as the only inflamma-
tory cytokine with elevated levels in a large proportion of HH patients, especially in those who were recently in
need of phlebotomy, suggesting a potential role for this
cytokine as a biomarker of disease severity. ζ-Globin
gene expression was highly upregulated in C282Y
homozygotes but not in H63D HH patients or H63D/ C282Y, suggesting its association with hemochromato-
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Statement of Ethics

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics, South East Region (2014/1732). Written information on the study was given to all patients and controls, and written informed consent was obtained from all study participants.

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

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