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MicroRNA Expression Analysis in Serum of Patients with Congenital Hemochromatosis and Age-Related Macular Degeneration (AMD)

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Congenital hemochromatosis is a disorder caused by mutations of genes involved in iron metabolism, leading to increased levels of iron concentration in tissues and serum. High concentrations of iron can lead to the development of AMD. The aim of this study was to analyze circulating miRNAs in the serum of congenital hemochromatosis patients with AMD and their correlation with the expression of genes involved in iron metabolism.

Material/Methods: Peripheral blood monolayer cells and serum were obtained from patients with congenital hemochromatosis, congenital hemochromatosis and AMD, AMD patients without congenital hemochromatosis, and healthy controls. Serum miRNAs expressions were analyzed by RT-PCR (qRT-PCR) using TaqMan MicroRNA probes, and proteins levels were measured by ELSA kits. Gene polymorphisms in *TF* and *TFRC* genes were determined using the TaqMan discrimination assay.

Results: Statistical analysis of the miRNAs expressions selected for further study the miR-31, miR-133a, miR-141, miR-145, miR-149, and miR-182, which are involved in the posttranscriptional expression of iron-related genes: *TF, TFRI, DMT1, FTL,* and *FPN1*. It was discovered that the observed changes in the expressions of the miRNAs was correlated with the level of protein in the serum of the analyzed genes. There were no statistically significant differences in the distribution of genotype and allele frequencies in *TF* and *TFRC* genes between analyzed groups of patients.

Conclusions: The differences studied in the miRNA serum profile, in conjunction with the changes in the analyzed protein levels, may be useful in the early detection of congenital hemochromatosis in patients who may develop AMD disease.

MeSH Keywords: **Iron Metabolism Disorders • Macular Degeneration • Transcriptome**

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Background

Congenital hemochromatosis is the most common genetic autosomal recessive disease among Europeans, occurring in 1 to 200–400 inhabitants of northwestern Europe [1]. Characterized by a genetic abnormal iron economy, it is the result of the *HFE* gene mutation (which increases the absorption of iron and results in its accumulation in tissues) or the mutations of other genes such as *HAMP*, *HJV*, *TfR2*, or *FPN1*. Excessive intestinal absorption or impaired circulation are responsible for the increased intercellular iron storage in various tissues, which leads to their failure. The most vulnerable to these effects are the liver, pancreas, and heart [2]. Hemochromatosis is also the most common hereditary metabolic disease of the liver. In most cases, if left untreated, it causes cirrhosis of the liver. Approximately 6% of patients develop primary hepatocellular carcinoma. Hemochromatosis also leads to cardiomyopathy, diabetes, hypothyroidism, hypogonadotropic hypogonadism, impotence, and arthropathy. Iron is involved in many important metabolic processes, such as electron transport, oxygen delivery, enzyme and coenzyme activity, and DNA synthesis. In addition, excess of iron may lead to the generation of iron-mediated reactive oxygen species and contribute to the formation of cellular alterations at the molecular level, including gene mutations. Excess iron increases the risk of developing hemochromatosis by initiating the formation of free radicals and promoting the oxidation of DNA [3]. The high concentration of iron causes oxidative stress to the secondary function of and structural damage to the organs [4,5]. Oxidative stress is involved in the pathogenesis of many chronic disorders, including inflammatory and neurologic diseases.

Reactive oxygen species (ROS) may play a role in age-related macular degeneration (AMD) [6]. Age-related macular degeneration is an ophthalmic disorder, and is currently one of the most important public health problems worldwide. It is the primary cause of blindness in developed countries, characterized by degenerative processes in the central part of the retina, the macula. AMD can take the form of dry (atrophic) or exudative. The etiology of AMD is multifactorial. Besides the environment, genetic factors and epigenetic mechanisms are relevant to AMD pathogenesis. It is believed that oxidative stress plays an important role in AMD development. One of the sources of oxidative stress in the macula could be iron ions, which are capable of promoting hydroxyl radical generation through the Fenton reaction. Some patients with hemochromatosis develop AMD. The diversity and non-specificity of symptoms make it difficult to diagnose. Therefore, recent studies have been performed to provide efficient biomarkers for early recognition of AMD in patients with congenital hemochromatosis, which could be used in screening programs in the future.

It was recently shown in the modulation of gene-expressing profiles that there are engaged, short, single-stranded, non-coding molecules called microRNAs (miRNAs). In the cytoplasm, miRNAs initiate the formation of miRNA-RNA silencing complexes by binding to the 3'-UTRs of target transcripts, leading to the destabilization of miRNAs or the repression of the translation process. miRNAs play a significant role in many fundamental cellular processes, such as cell differentiation, proliferation, phagocytosis, inflammation, and apoptosis. miRNAs may be involved in ophthalmology diseases such as retinitis pigmentosa [7], retinoblastoma [8], and ocular neovascularization [9,10] and may be useful as potential biomarkers of AMD [11].

The aims of this study were: (1) to assess the expression levels of miRNA genes that are involved in the posttranscriptional expression of iron-related genes in patients with congenital hemochromatosis with AMD versus AMD patients without hemochromatosis, and healthy controls. (2) To investigate whether the alterations in the expression profiles of selected miRNAs appear and are characteristic for AMD patients with congenital hemochromatosis. (3) To find a correlation between miRNAs and iron metabolism protein levels in serum. (4) To analyze the distribution of genotypes and alleles of a few functional SNPs in iron metabolism genes in patients with and without congenital hemochromatosis and attempt to find a correlation with the level of protein in the serum.

To reach these goals, blood samples from patients with congenital hemochromatosis, with and without AMD, and healthy controls were collected. The expression levels of miRNAs gene panels were assessed by quantitative RT-PCR analysis. The expression patterns of various miRNAs in serum were screened using TaqMan® Human MicroRNA Array A (Applied Biosystems), followed by an extensive self-validated study using individual quantitative RT-PCR (qRT-PCR) assays. Iron-dependent gene expressions on the protein level in serum were assessed by ELISA tests.

Material and Methods

Collection of biological samples

Blood sample collections were performed in accordance with the protocols approved by the Committee of Bioethics at the Medical University of Lodz (RNN/228/11/KE), and informed consent was obtained in accordance with the Declaration of Helsinki. From patients hospitalized in the Military Teaching Hospital No. 2 at the Medical University in Lodz, Poland and the Sal-Med Medical Center, Lodz, Poland, we obtained 25 samples from patients with congenital hemochromatosis and AMD, 100 samples from AMD patients without congenital hemochromatosis, and 100 samples from healthy controls. Patients with congenital hemochromatosis were diagnosed

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Table 1. Characteristics of AMD patients with and without congenital hemochromatosis and controls.

		AMD patients without Congenital hemochromatosis		AMD patients with Congenital hemochromatosis		patients with Congenital hemochromatosis		Controls	
Residence	Village	26	(26%)	8	(32%)	10	(40%)	29	(29%)
	City	74	(74%)	17	(68%)	15	(60%)	71	(71%)
Property status	Unsatisfactory	35	(35%)	10	(40%)	10	(40%)	40	(40%)
	Satisfactory	53	(53%)	12	(48%)	9	(36%)	45	(45%)
	Good	12	(12%)	3	(10%)	6	(24%)	15	(15%)
Smoking status	Non-smoking	60	(60%)	12	(48%)	12	(46)%	120	(51)%
	Smoking (≤1P/D)	24	(24%)	10	(40%)	9	(38%)	50	(38%)
	Smoking (≥1P/D)	16	(16%)	3	(12%)	4	(16%)	30	(11%)
Age (standard deviation and range)		70.1	(11.2)	68.5	(9.5)	71%	(8.2)	67.6	(10.1)
BMI (body mass idex)		27.3		27.2		25.9		27.5	

due to their symptoms at the Clinical Genetics Department of the Medical University of Lodz.

The research materials analyzed were the peripheral blood monolayer cells (PMBC) and serum. The serum and PMBCs were isolated from all blood samples according to Szemraj et al. [11]. The characteristics of patients are presented in Table 1. All samples were stored at -80°C

Patient inclusion criteria

Written consent was obtained from each patient before enrolment into the study. Healthy volunteers without ocular abnormality and congenital hemochromatosis served as controls. No statistically significant differences with regard to sex or age were observed between the group of patients and the healthy controls.

Patient exclusion criteria

Patients were excluded from the study on the basis of the following: a diagnosed acute eye inflammation, chronic inflammation, diabetes, polyarthritis reumatoidea, and a body temperature above 38°C for at least 2 weeks, as well as chronic consumption of alcohol, nicotine, or narcotics. In addition, pregnant and lactescent women were also excluded from the study on the basis of ethical and legal requirements.

RNA extraction

miRNAs were isolated from 400-µl samples of serum taken from patients with congenital hemochromatosis, congenital hemochromatosis with AMD, AMD without hemochromatosis, and healthy controls, using the *mir*Vana PARIS kit (Ambion) according to the manufacturer's protocol [11]. The amount and quality of the isolated RNA was checked with the Agilent RNA 6000 Nano kit in accordance with the manufacturer's recommendations using the 2100 Bioanalyzer (Agilent Technologies). Complementary DNA (cDNA) was transcribed from RNA using the TaqMan® RNA Reverse Transcription kit (Applied Biosystems).

Screening of congenital hemochromatosis with AMD, and AMD without congenital hemochromatosis samples for associated miRNA genes.

Reverse transcription of isolated miRNA from 5 patients with congenital hemochromatosis and AMD, 5 AMD patients without hemochromatosis, and 5 healthy controls were prepared according to the manufacturer's recommendation using the Megaplex™RT Primers Human Pool A and B, and the TaqMan® Human MicroRNA Array A and B purchased from Applied Biosystems using a 7900 HT System (Applied Biosystems). The expression levels of 377 human miRNA genes for each group were analyzed. miRNA genes that revealed an altered expression profile and were homological to the 3'-UTRs of iron-related genes according to the DIANA database, were chosen for further investigations.

RT-PCR of selected miRNAs

miRNA conversion to cDNA was performed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). The samples were incubated (30 min at 16°C and 30 min at 42°C) in a thermocycler (Biometra). Reverse transcriptase was inactivated (5 min at 85°C) and the obtained cDNA was stored at –80°C. We used 5 nmol mirVana miRNA Mimic (cel-miR-39) as an endogenous control (Ambion, Austin, TX) for normal reverse transcription conditions. Each target probe was amplified in a separate 96-well plate. All samples

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were incubated at 50°C for 2 min and at 95°C for 10 min, and then cycled at 95°C for 30 s, at 60°C for 30 s, and at 72°C for 1 min; 40 cycles were performed. Fluorescence emission data were captured and miRNA levels were quantified using the critical threshold (Ct) value in total. Analyses were performed with the ABI Prism 7000 (SDS Software). Each assay included controls without RT and with no template cDNA. Relative gene expression levels were obtained using the ΔΔCt standard 2–ΔΔct calculations and expressed as fold change of the control sample [12]. Amplification specific transcripts were further confirmed by obtaining melting curve profiles.

Determination of serum TF, TFRI, DMT1, FTL, and FPN1 protein levels using ELISA

For the quantitative detection of circulating serum TF, TFRI, DMT1, FTL, and FPN1 proteins, we used the Human TF ELISA from Thermo Fischer Scientific (Waltham, MA USA), the Human TFR1 ELISA from Biocompare (San Francisco CA, USA), the Human DMT1 ELISA from Biocompare (San Francisco CA, USA), the Human FTL ELISA from MyBiosource (San Diego CA, USA), and the Human FPN1 ELISA from MyBiosource (San Diego CA, USA). Each serum sample was analyzed 3 times. The instructions and the calculations of results were performed according to the manufacturer's recommendations. β-actin was used for the endogenous control of protein concentration in the samples and detected using the Human Actin Beta (ACTb) ELISA kit (BMASSAY). The color development was stopped (Stop Solution) and the intensity of the color was measured by the Thermo Labsystems Multiskan Ascent 354 from Lab Recyclers at 450 nm.

Determination of ferrum level in serum using colorimetric method

The ferrum concentration in each serum sample was measured by colorimetric methods using the QuantiChrom Iron Assay kit (Bioassay Systems, Hayward, CA) at 450 nm according to manufacturer's recommendations on the PowerWave XS (Bio-Tek Instruments, Winooski, VT).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes by the standard procedure according to manufacturer's protocol (A&A Biotechnology, Gdańsk, Poland). The amount and quality of the isolated DNA was analyzed via Nanodrop (Thermo Fisher Scientific Inc, De, USA). The genotypes of polymorphisms were identified, as reported in a previous study [13], by research assistants blinded to the clinical status using the TaqMan discrimination assay.

The reaction mixture (5 μl) contained DNA (10 ng), TaqMan Genotyping Master Mix (Applied Biosystems Inc. Carlsbad, CA), and FAM and VIC probes (200 nM) and primers specific for TaqMan SNP (*TF*) assay code C_326218248_10 for rs8177178, C_27915079_20 for rs4481157, TaqMan SNP (*TFRC*) assay code C_3259537_10 for rs3817672, and C_2184549_20 for rs2075674 (Applied Biosystems). The PCR reaction was performed on the 7900 HT Real-Time PCR System (Applied Biosystems) for 15 min at 95°C, followed by 30 cycles of following steps: 15 s at 92°C and 1 min at 60°C. Allelic discrimination was performed on the post-PCR product. Analyses of amplification products were performed using SDS software, version 1.2. The efficiency of amplification was calculated from the slope of the standard curves generated by individually screening each probe when testing diluted heterozygote samples. A sample without a template was used as a negative control.

Statistical analysis

The Wilcoxon matched-pairs test was used to compare groups of dependent continuous variables: RQ levels of miRNAs at 2 different timepoints. Spearman rank correlation coefficient (R) was used to compare independent variables: regeneration and miRNA's RQ level. Comparisons and correlations were considered significant at $p<0.05$.

Results

Sample collection.

Research groups: Patients with the congenital hemochromatosis dry form of AMD with or without congenital hemochromatosis and controls were selected on the basis of age and sex: 55 female and 45 male AMD patients without congenital hemochromatosis, 13 female and 12 male patients with congenital hemochromatosis and AMD, 11 female and 14 male patients with congenital hemochromatosis, and 60 female and 40 male patient controls were selected. No significant differences were observed with regard to sex (p=0.24), age (p=0.51), differences in residence (p=0.16), status (p=0.70), smoking status (p=0.40), or BMI index (p=0.42) between patients with congenital hemochromatosis and both groups of AMD patients versus the control group. Between the groups of AMD patients, no significant differences were seen: sex (p=0.37), age (p=0.44), differences in residence (p=0.19), property status (p=0.53), smoking status (p=0.44), and BMI index (p=0.37).

Analysis of miRNA panel in serum of AMD patients with and without congenital hemochromatosis in comparison to healthy controls.

Previous studies have reported that some serum miRNAs are overexpressed in AMD patients [11]. To screen for miRNAs associated with iron import, storage, and AMD risk, 5 AMD patients

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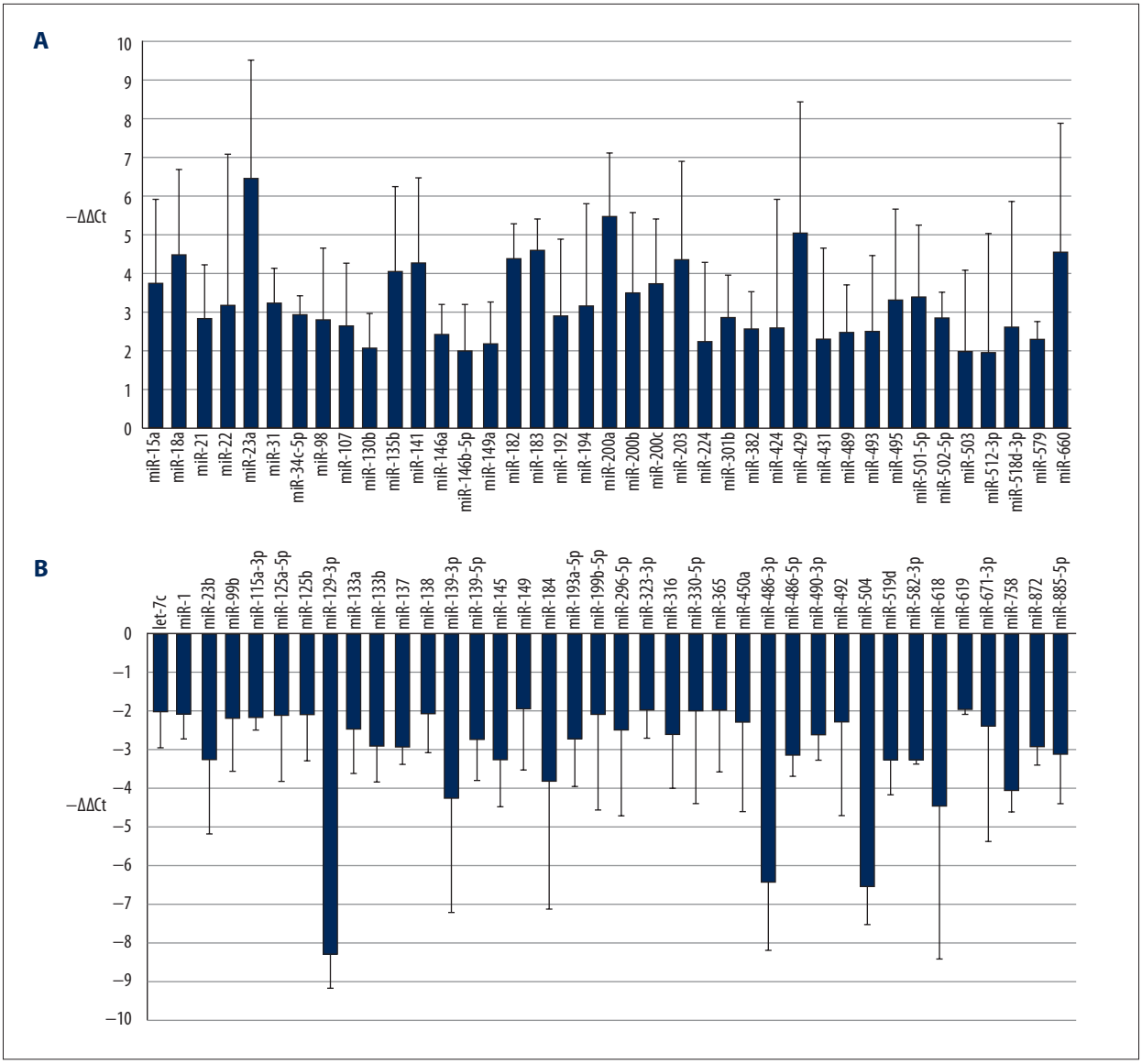

Figure 1. Panel of miRNA expression in serum. (**A**) Up-regulated and (**B**) Down-regulated in congenital hemochromatosis patients with AMD in comparison to average control sample value. Data are presented as mean ±SE of ΔΔ Ct values; n=5.

with congenital hemochromatosis, 5 AMD patients without congenital hemochromatosis, 5 congenital hemochromatosis patients, and 5 healthy controls without AMD and congenital hemochromatosis were analyzed. The expression levels of 377 miRNA genes were measured with TaqMan human miRNA arrays. Approximately 20% of analyzed miRNAs revealed alterations in their gene expression profile (–2>ΔΔCt>2), of which 39 were overexpressed and 38 were underexpressed in AMD patients with congenital hemochromatosis in comparison to controls (Figure 1). We found altered expression of 38 miRNAs in AMD patients without congenital hemochromatosis. The differences in miRNA expressions between AMD patients with and without congenital hemochromatosis and the controls were highly significant (expression was not observed in controls) ($p<0.0001$). Twenty-four of these miRNAs showed at least a 10-fold change in relative expression rate. Among the genes with altered expression profiles in AMD patients with and without congenital hemochromatosis, miR-31, miR-133a, miR-141, miR-145, miR-149, miR-182, miR-194, and miR-758 showed homology to 3'UTRs of iron-related genes according to the DIANA database (Figure 2), and were selected for further investigations.

Expression profile of selected miRNA genes in AMD patients with and without congenital hemochromatosis

The RT-QPCR assay revealed that all selected miRNAs were detectable in tested materials and the difference between miRNA

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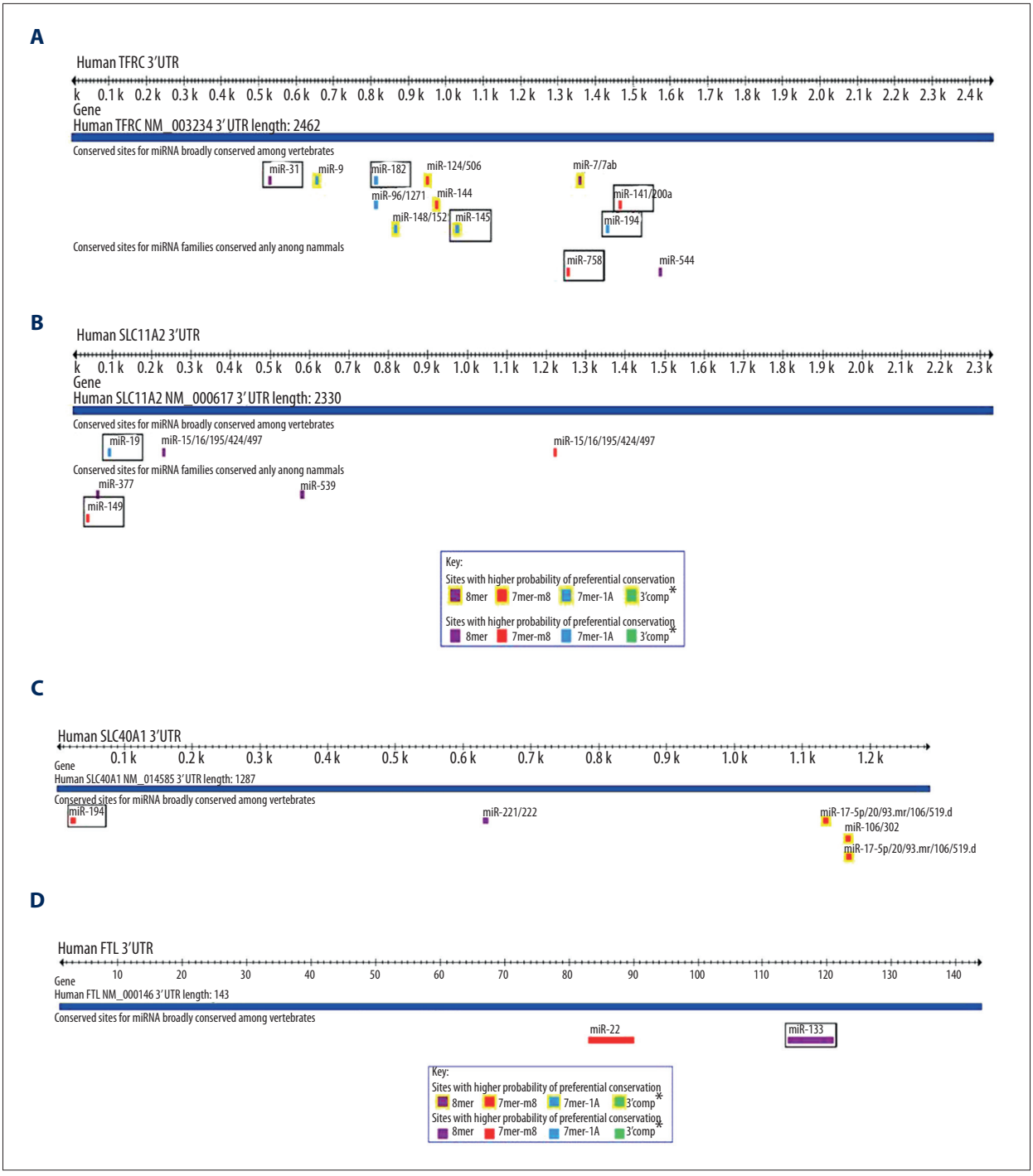

Figure 2. Homology between sequences of miRNA and 3-UTRs of iron genes: (**A**) *TFRC (TFRI)*, (**B**) *SLC11A2* (DMT1), (**C**) *FTL*, (**D**). *SLC40A1 (FPN1)*, according to the DIANA database; 8mer (purple squares), 7mer-m8 (red squares), 7mer-1A (blue squares), 3'comp (green squares); squares with yellow borders symbolize sites with higher probability of preferential conservation; miRNA genes selected for further analysis are framed.

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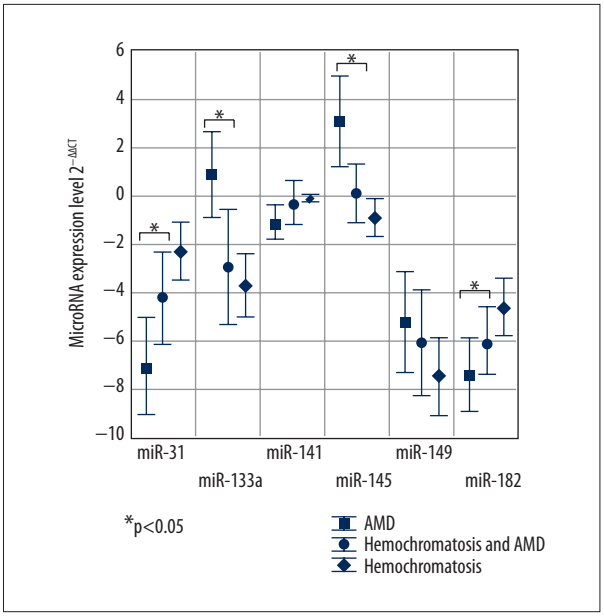

Figure 3. Expression of miR-31, miR-133a, miR-141, miR-145, miR-149, and miR-182 in serum of congenital hemochromatosis patients, congenital hemochromatosis patients with AMD, and AMD patients. Data are presented as mean ±SE for expression levels of analyzed miRNA calculated as $2^{-\Delta\Delta Ct}$. Significance of differences between patients and controls was estimated with the paired *t* test. Significance of differences among groups was estimated with one-way ANOVA followed by Tukey's test.

expression levels between AMD patients with and without congenital hemochromatosis was highly significant ($p<0.001$) for most genes, except miR-758 and miR-194, in which the levels of miRNA remained almost the same. The average expression levels of miR-31 (1.69x), miR-141 (3.6x), and miR-182 (1.23x) in serum of AMD patients with congenital hemochromatosis were significantly enhanced, while miR-133a (1.31x), miR-145 (31x), and miR-149 (1.17x) demonstrated diminished expression, compared to AMD patients without congenital hemochromatosis. Statistically significant differences ($p<0.05$) were found by analyzing the expressions of miR-31, miR-133a, miR-145, and miR-182 (Figure 3).

Expression profiles of selected genes, *TF*, *TFRC*, *DMT1*, *FTL*, *FPN1*, on protein level in serum, iron level of AMD patients with and without congenital hemochromatosis.

Selected miRNAs showed homology to the 3'UTRs of iron-related genes. In our study, we decided to analyze the *TF, TFRC, DMT1, FTL,* and *FPN1* gene expressions at the protein level in serum of AMD patients with and without congenital hemochromatosis (Figure 4). The averaged values of protein concentration in serum expressed in% are shown in Table 2. Data analysis showed about a 24.8%, 22.3%, 19.3%, and 50.7% increase of FTL, transferrin receptor (TFRC), transferrin, and DMTI protein levels, respectively, in those AMD patients with hemochromatosis versus AMD patients without hemochromatosis, while the level of ferroportin decreased by about 50.7%. It was also observed that serum iron concentration increased by about 15.6% in AMD patients with hemochromatosis patients versus AMD patients without hemochromatosis.

Genotyping

We analyzed 2 generally recognized functional polymorphism sites (rs8177178 and rs4481157) in transferrin gene *TF,* and 2 sites (rs3817672 and rs2075674) in transferrin receptor gene *TFRC*. Based on the results, we found no statistically significant differences in the distribution of genotype and allele frequencies between the groups of hemochromatosis patients with AMD and those with only AMD. The distribution of genotypes and alleles of the rs8177178 and rs4481157 *TF* genes, and the rs3817672 and rs2075674 *TFRC* genes, are presented in Table 3. The statistical analysis showed no correlation between levels of TF and TFRC proteins in serum in those hemochromatosis patients with AMD and those with just AMD.

Distribution of genotypes and alleles of the rs8177178 and rs4481157 *TF* genes, and rs3817672 and rs2075674 *TFRC* genes. OR, odds ratio; 95% CI, 95% confidence interval, *p*-value ≤0.05 was accepted as the level of statistical significance.

Correlation between relative expression levels of genes related to iron metabolism in hemochromatosis patients with AMD compared to AMD patients without hemochromatosis

It has been suggested that there is an additional metabolic regulatory mechanism of iron-dependent miRNA. To clarify whether it operates in hemochromatosis patients with AMD, the expression level of the miRNA homologous 3'UTR regions of genes involved in the metabolism of iron ions needs to correlate with the level of expression of the target genes. The correlation coefficients of hemochromatosis patients with AMD show a positive correlation between *TRFC* gene expression and the expression of miR 31 ($r=0.87$ $p<0.001$) and a negative correlation for the miR-145 ($r=-0.36$ $p=0.026$) in serum. However, no correlation with *TRFC* gene expression was observed for patients with just AMD ($r=0.66$ $p=0.151$ and $r=0.12$ $p=0.847$ for miR 31 and miR-145, respectively). There were no correlations observed between other analyzed miRNA genes and their target genes, both in congenital hemochromatosis patients with and without AMD, and AMD patients.

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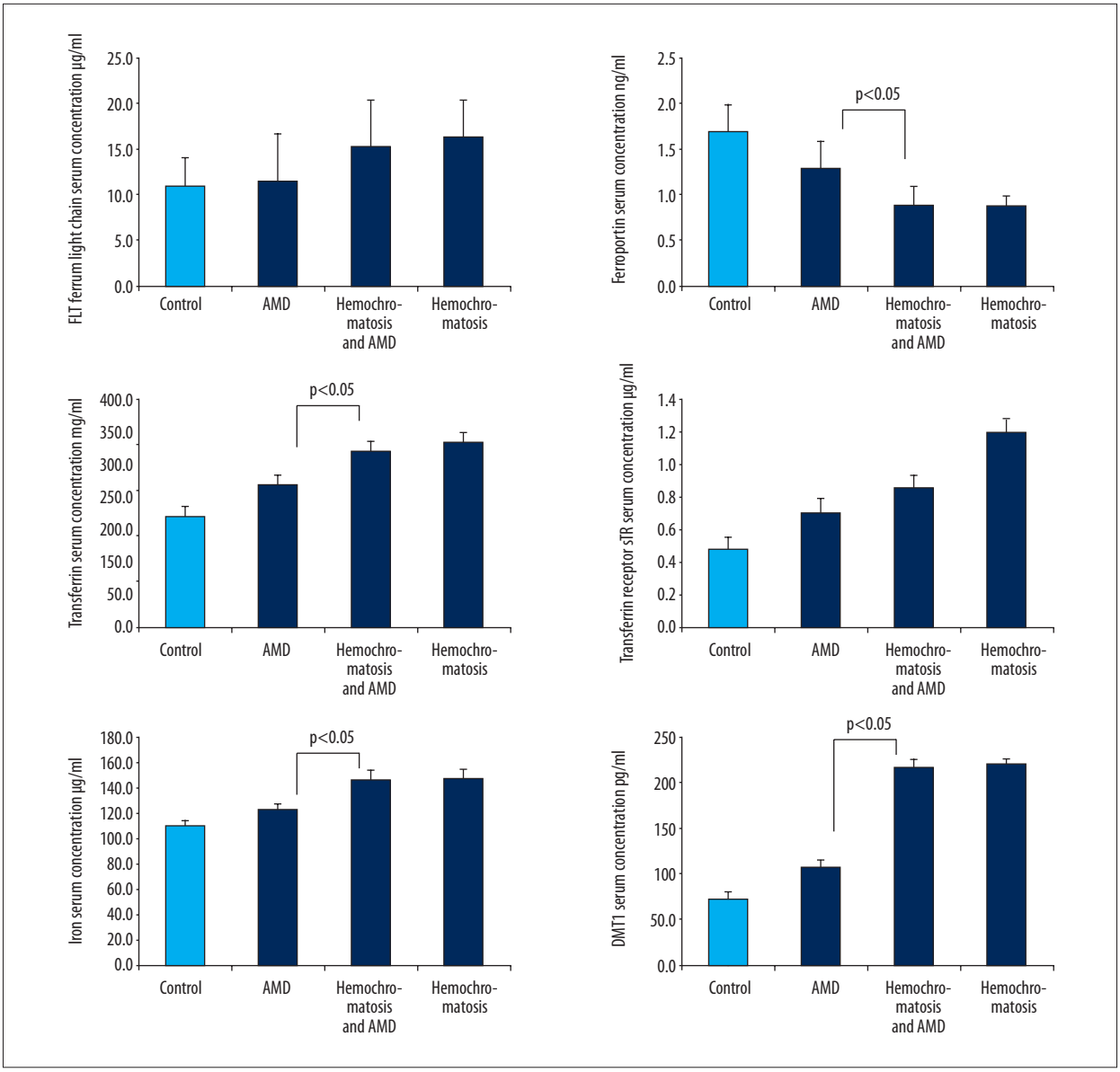

Figure 4. Expression of FTL, FPN1, TFRC, and DMT1 proteins and iron level in serum of controls, congenital hemochromatosis patients, congenital hemochromatosis patients with AMD, and AMD patients. Data are presented as mean ±SE for protein and iron levels. Significance of differences between patients and controls was estimated with the paired *t* test. Significance of differences among groups was estimated with one-way ANOVA followed by Tukey's test

Table 2. Average percent changes in *TFRC (TFRI), SLC11A2 (DMT1), FTL, SLC40A1(FPN1)* gene expression at the protein level and iron concentration in AMD and AMD with Hemochromatosis patients serum versus control and Hemochromatosis patients.

	FTL (% increase)		Feroportin (% decrease)		Iron (% increase)		Transferin receptor (% increase)		Transferin (% increase)		DMT1 (% increase)	
	AMD	AMD-hemoch	AMD	AMD-hemoch	AMD	AMD-hemoch	AMD	AMD-hemoch	AMD	AMD-hemoch	AMD	AMD-hemoch
Control	6.08%	29.4%	31%	69.7%	10.6%	24.6%	40%	80%	28.3%	59%	47.8%	51.5%
Hemochromatosis	69.7%	6.1%	89%	0%	16%	1.2%	74.1%	33%	23%	9.6%	199%	1.7%

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Table 3. Distribution of genotypes and alleles of the rs8177178 and rs4481157 *TF* genes, rs3817672 and rs2075674 *TFRC* genes. OR, odds ratio; 95% CI, 95% confidence interval, *p*-value ≤0.05 was accepted as the level of statistical significance.

Genotype/Allele rs8177178 *TF*	Control N=100 N (%)	AMD N=100 N (%)	Hemochromatosis with AMD N=25 N (%)	OR (95% CI)	*p*
GG	43 (43)	44 (44)	11 (45)	1.05 (0.58–1.25)	0.605
GA	45 (45)	45 (45)	10 (42)	0.86 (0.68–1.18)	0.768
AA	12 (12)	11 (11)	4 (13)	1.09 (0.59–1.31)	0.732
G	66 (66)	66 (66)	17 (67)	0.99 (0.77–1.41)	0.579
A	34 (34)	34 (34)	8 (33)	0.96 (0.68–1.21)	0.579
Genotype/Allele rs4481157 *TF*	**Control N=100 N (%)**	**AMD N=100 N (%)**	**Hemochromatosis with AMD N=25 N (%)**	**OR (95%CI)**	***p***
GG	23 (23)	19 (19)	6 (26)	0.97 (0.59–1.47)	0.961
AG	47 (47)	61 (61)	12 (47)	1.09 (0.67–1.46)	0.856
AA	30 (30)	20 (20)	7 (27)	0.95 (0.59–1.51)	0.869
G	49 (49)	49 (49)	12 (48)	1.09 (0.71–1.27)	0.937
A	51 (51)	51 (51)	13 (52)	0.93 (0.78–1.26)	0.937
Genotype/Allele rs3817672 *TFRC*	**Control N=100 N (%)**	**AMD N=100 N (%)**	**Hemochromatosis with AMD N=25 N (%)**	**OR (95%CI)**	***p***
AA	30 (30)	36 (36)	9 (35)	1.19 (0.74–1.58)	0.597
GA	56 (56)	47 (47)	11 (45)	1.07 (0.67–1.49)	0.305
GG	14 (14)	17 (17)	5 (20)	0.68 (0.51–1.21)	0.467
A	59 (59)	58 (58)	15 (58)	1.23 (0.92–1.45)	0.099
G	41 (41)	42 (42)	10 (42)	0.80 (0.64–1.11)	0.099
Genotype/Allele rs2075674 *TFRC*	**Control N=100 N (%)**	**AMD N=100 N (%)**	**Hemochromatosis with AMD N=25 N (%)**	**OR (95%CI)**	***p***
GG	36 (36)	44 (44)	12 (46)	1.23 (0.71–1.58)	0.961
GA	40 (40)	35 (35)	7 (28)	0.94 (0.57–1.45)	0.264
AA	24 (24)	21 (21)	6 (23)	0.81 (0.52–1.12)	0.149
G	56 (56)	63 (63)	16 (62)	1.26 (0.77–1.73)	0.417
A	44 (44)	37 (37)	9 (38)	0.90 (0.61–1.16)	0.417

Discussion

Age-related macular degeneration is a chronic disease leading to progressive central vision loss. It is a result of photoreceptors and retinal pigment epithelium degeneration and has multifactorial etiology. It is believed that oxidative stress and inflammation are the most important factors in the pathogenesis of AMD [14–18]. Data suggest that iron ions take part in the induction of oxidative stress in the retina, and the gradual increase of this stress may be an important risk factor of AMD [19,20]. In retinas of patients with AMD obtained postmortem, deposits of iron were found that were not present in the retinas of patients without AMD [21]. A solution of iron sulfate or hemoglobin, injected into the vitreous body of the rabbit eye, resulted in invasive retinal cells hypertrophy, especially the retinal pigment epithelium layer [22].

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Hemochromatosis is an example of a pathological condition that results from disorders related to iron hemostasis. It is a genetically determined heterogeneous disorder leading to a chronic accumulation of iron in body tissues [23]. In hemochromatosis, iron ions gradually accumulate in cells of the retinal pigment epithelium [24,25].

The link between the genes *HFE, HJV, HAMP*, and *SLC40A1 TFR2*, and pathological process of iron accumulation allows us to assume that they all take part in this chronic process. It is believed that the classic form of the disease most commonly causes 2 mutations, C282Y and H63D, in the *HFE* gene. However, data show the presence of other mutations in different genes, which may also predispose to an increased absorption of iron from the digestive tract, which is then deposited into various tissues. Introduced in 1996, genetic testing has resulted in an important improvement in the diagnosis of congenital hemochromatosis. It has enabled the diagnosis of the disease in the early stages, before there has been an accumulation of iron that causes irreversible organ damage. Checking the balance of iron in the body requires communication between the places of its collection, use, and storage. Proteins involved in the metabolism of iron ions are typically synthesized by hepatocytes, but their levels vary in different diseases [26]. Gene expression is regulated by specific miRNAs. miRNAs are involved in the posttranscriptional regulation of almost 60% of coding genes responsible for clinical functions of the body, such as proliferation, differentiation, cellular homeostasis, and carcinogenesis. Currently, more is known about the expression of miRNAs involved in the regulation of gene expression and their involvement in AMD pathology [27]. Few studies have analyzed circulating miRNA dysregulation in AMD [11,28–30].

The aim of this study was to assess the expression levels of over 14 iron-related genes from patients with congenital hemochromatosis with and without AMD, AMD patients without hemochromatosis, and healthy controls. Among these, there are structural genes (*TF, TFRC, DMT1, FTL, FPN1*) and miRNAs (miR-19a, miR-31, miR-133a, miR-141, miR-145, miR-149, miR-182, miR-194, miR-758) that are involved in their post-transcriptional regulation. All analyzed genes may contribute to the excessive accumulation of iron ions in cells, leading to the initiation and development of various diseases, including AMD. Increased levels of iron ions lead to the formation of ROS that are involved in the development of gene mutations.

In this study, we began by analyzing over 350 human miRNA expressions in a small patient population (5 patients from each group). Differences in gene expression between congenital hemochromatosis patients with AMD *vs.* AMD patients without hemochromatosis were observed in over 80 miRNAs. Then, the observed differences in the genes expression were confirmed in the remaining group of patients. We selected for further study those miRNAs that were homologous to the 3'UTR regions of iron-related genes, according to the information contained in the miRNA DIANA database. The sequences of the selected miRNAs were homological to the *TFRC, DMT1, FTL,* and *FPN1* genes, which encode proteins involved in cellular iron transport and storage.

It has been shown that miR-31, miR-141, miR-182, and miR-194 are overexpressed, and miR-133a, miR-145, and miR-149 have a decreased level of expression in patients with congenital hemochromatosis and AMD in comparison to AMD patients without hemochromatosis. All of the analyzed genes were detected in serum of patients with congenital hemochromatosis with and without AMD, and AMD patients without hemochromatosis, but not in controls. The analysis of *TFRC, DMT1, FTL,* and *FPN1* gene expressions and the protein levels in serum showed significant differences in expression profiles between the analyzed groups. The expression of the analyzed *FTL, TFRC, TF,* and *DMTI* genes increased from control to the hemochromatosis without AMD group, whereas the opposite trend was observed for ferroportin protein. Iron concentration in serum was also changed. The highest levels were observed in patients with congenital hemochromatosis without AMD and in patients with congenital hemochromatosis with AMD, whereas the lowest was observed in the control group. High serum ferritin levels can be explained by excessive absorption of iron by the cells. Absorption of iron by cells depends on DMT1 and TFR1, while the release from cells is mediated by ferroportin [31].

In this study, *FPN1* gene expression levels were estimated and demonstrated to considerably increase. To determine the expression levels of the FPN1 protein, we also estimated the concentration of ferroportin in patients with congenital hemochromatosis with AMD *vs.* AMD patients without hemochromatosis. We showed that the level of analyzed proteins was significantly lower in AMD patients with congenital hemochromatosis compared to AMD patients without hemochromatosis. The results indicate the overexpression of genes responsible for the collection of iron ions (DMT1 and TFR1) and a reduction in the level of ferroportin, leading to a decrease in the exportation of the metal ions. This may contribute to the excessive iron accumulation in cells. Consequences of this iron accumulation could include the production of free radicals, leading to genetic mutations. In this study, for the first time, we demonstrated that the *FPN1* gene expression is correlated with miR-194 expression, which leads to a reduction in the level of ferroportin in patients with congenital hemochromatosis and AMD. miR-194 may bind to the 3 'UTR region of *FPN1* transcript and inhibit the translation process.

Analysis of data in the literature was the basis for further exploration of predisposing factors of AMD development in patients with hemochromatosis. Genetic factors play an important

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role in the pathogenesis of AMD. Gene variants have been identified that significantly affect the risk of development of AMD [32–35]; therefore, we analyzed the genetic variation in the genes as the key factor of iron homeostasis. Our study included genes encoding the transferrin receptor and transferrin. Both proteins are part of the transportation system of iron in the blood stream. We analyzed 4 polymorphic sites: 2 in the *TF* gene and 2 in the *TRFC* gene. The distributions of the genotype and allele frequencies of the analyzed polymorphic sites in congenital hemochromatosis patients with AMD versus AMD patients without hemochromatosis showed no statistically significant changes. The statistical analysis showed no correlation between the levels of TF, TFRC, and iron and the genotype of the studied genes in patients with congenital hemochromatosis and AMD. The obtained results suggest the role of circulating miRNAs in blood in the regulation of *TF* and *TRFC* gene expression at the protein level and their levels in the serum of congenital hemochromatosis patients with AMD.

Conclusions

Our results suggest that transferrin and the transferrin receptor, together with the studied miRNAs, influence the risk of developing AMD for patients with congenital hemochromatosis. Additional experiments are needed to confirm our findings.

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