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ORIGINAL RESEARCH

Distinctive Under-Expression Profile of Inflammatory and Redox Genes in the Blood of Elderly Patients with Cardiovascular Disease

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Purpose: Chronic low-grade inflammation and oxidative stress are present in most of the pathologic mechanisms underlying non-communicable diseases. Inflammation and redox biomarkers might therefore have a value in disease prognosis and therapy response. In this context, we performed a case–control study for assessing in whole blood the expression profile of inflammation and redox-related genes in elderly subjects with various comorbidities.

Patients and Methods: In the blood of 130 elderly subjects with various pathologies (cardiovascular disease, hypertension, dyslipidemia including hypercholesterolemia, type 2 diabetes mellitus), kept under control by polyvalent disease-specific medication, we investigated by pathway-focused qRT-PCR a panel comprising 84 inflammation-related and 84 redox-related genes.

Results: The study highlights a distinctive expression profile of genes critically involved in NF-κB-mediated inflammation and redox signaling in the blood of patients with cardiovascular disease, characterized by significant down-regulation of the genes *NFKB2, NFKBIA, RELA, RELB, AKT1, IRF1, STAT1, CD40, LTA, TRAF2, PTGS1, ALOX12, DUOX1, DUOX2, MPO, GSR, TXNRD2, HSPA1A, MSRA,* and *PDLIM1*. This gene expression profile defines the transcriptional status of blood leukocytes in stable disease under medication control, without discriminating between disease- and therapy-related changes.

Conclusion: The study brings preliminary proof on a minimally invasive strategy for monitoring disease in patients with cardiovascular pathology, from the point of view of inflammation or redox dysregulation in whole blood.

Keywords: aging-related diseases, cardiovascular disease, inflammation, NF-κB signaling, redox metabolism, oxidative stress

Introduction

The growing prevalence of non-communicable diseases (NCDs), especially in elderly individuals, determines the need for an accelerated search of biomarkers that could improve disease prognosis, monitoring, and therapeutic engagement. Extensive evidence indicates a critical role of low-grade chronic inflammation and oxidative stress as active participants in the pathological mechanisms underlying NCDs such as cardiovascular disease (CVD), hypertension, type 2 diabetes mellitus (T2DM), and dyslipidemia.[1–5] Underlying mechanisms are cellular senescence,[6] mitochondrial dysfunction,[7] defective autophagy and mitophagy of misfolded or oxidized proteins,[8] activation of inflammasomes by cell debris and misplaced "self" molecules,[9] dysregulation of the ubiquitin–proteasome system,[10] activation of the DNA damage response[11], and dysbiosis.[12]

A multitude of stimuli can trigger chronic inflammatory responses in innate immune cells, mainly in macrophages through cognate receptors of Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) that are signaling mostly via the NF-κB pathway.[1,13] A persistently increased NF-κB activity can drive senescence of immune cells that acquire an altered transcriptional phenotype, resulting in increased production of inflammatory factors such as IL-6, IL-8, IL-7, MCP-2, MIP-3, ICAM, Il-1α, and IL-β.[14] Concurrently, a persistent alteration of the redox metabolism occurring in elderly individuals, due to an increased production of reactive oxygen species (ROS) by mitochondrial and cytoplasmatic sources, or to defective antioxidant protection[15] is a risk factor for NCDs.[16] The ensuing low-grade redox dysregulation alters signaling responses to growth and trophic factors, cytokines, chemokines, etc., leading to cellular senescence and chronic inflammation.[17] Various comorbidities, such as obesity, hypertension, and T2DM contribute to exacerbate the deleterious action of this low-grade pro-inflammatory phenotype characterizing elderly individuals.[18]

The highly reactive circulating leukocytes reflect quite closely both systemic and localized inflammatory and redox-mediated mechanisms. Moreover, the recruitment of dysfunctional blood leukocytes into diseased tissues can amplify these pathological processes, hence worsening disease evolution.[19] Being easy to obtain from venous blood, the functional status of circulating leukocytes represents a window for detecting immune abnormalities in NCDs related to inflammation and redox metabolism.

This study aimed to identify gene expression changes that might underlie pathologic processes in elderly patients with various NCDs. We analyzed the expression of 168 inflammation- and redox-related genes in the blood of a cohort of 130 elderly patients with diseases kept under clinical management by polyvalent medication. The detected gene expression profile was consistent with a suppression of inflammatory and redox-mediated processes as adaptive mechanisms aimed at limiting disease progression or relapse.

Patients and Methods

Subjects

Elderly individuals (n=130), 67- to 81-year-old, were randomly recruited from 3 neurology, 1 psychiatry, and 2 geriatric clinics. The inclusion criterion was age above 60 years, and exclusion criteria comprised: (i) acute inflammatory reactions and infection in the last 30 days prior to the study inclusion; (ii) history of any type of cancer and autoimmune diseases; (iii) acute episodes of morbidities during the last year before being recruited in the present study. All the recruited subjects, complying with the above-mentioned inclusion/exclusion criteria, were clinically evaluated for the presence of NCDs. Patients presented one or several morbidities, comprising hypertension (HT), cardiovascular diseases (CVD), dyslipidemia (DL), including hypercholesterolemia (HC) and Type 2 diabetes mellitus (T2DM) as described in Table 1A. Disease was kept under control with polyvalent medication. For analyzing gene expression data, the subjects presenting a type of disease constituted the case group, while all the other subjects in the cohort, not presenting that specific disease, constituted the control group (Figure 1, Table 1B). The study was conducted in accordance with the Declaration of Helsinki, and was approved by the local ethics committees of participating clinics, as follows: Clinical Hospital Colentina, Bucharest, Romania – 12/11.05.2017; University Emergency Hospital, Bucharest, Romania – 56457/07.11.2017; "Prof. Dr. Al. Obregia" Psychiatry Clinical Hospital, Bucharest, Romania – 3/17.05.2017; "Ana Aslan" National Institute of Gerontology and Geriatrics, Bucharest, Romania – 299.1101.2018; Hospital Arnau de Vilanova de Lleida, Lleida, Spain – CE 1218. Written informed consent was obtained from all study participants at the moment of recruitment and blood collection.

Blood Samples

For all the recruited subjects, venous blood (2.5 mL) was collected in PAXgene Blood RNA Tubes (Qiagen) for RNA isolation and gene expression studies. Tubes with blood were kept at room temperature for 24 h, transferred to −20°C for another 24 h, and, finally, stored at −80°C until use.

For a subgroup of 46 elderly subjects, venous blood was collected in SSTII Advance vacutainers for serum separation and investigation of cardiovascular markers and several cytokines, chemokines, and growth factors in serum by xMAP protein multiplexing.

Gene Expression

Total RNA was isolated from the blood collected in PAXgene tubes according to the manufacturer's protocol. RNA purity and quantity were spectrophotometrically assessed using a NanoDrop 2000 equipment (Thermo Scientific). Reverse transcription was performed with the RT^2 First Strand Kit (Qiagen) using 400 ng of RNA. The expression of 84 key

Table 1 Socio-Demographic Features and Comorbidities in the Investigated Cohort of 130 Elderly Subjects (A) and in the Groups of Patients and Controls Classified According to Morbidities (B)

	A) The Cohort of 130 Elderly Subjects														
	Socio-demographic data														
	Age (mean ± SD)			74.4 ± 7.1											
	Gender (% females)			46.9%											
	Education in years (mean ± SD)			10.0 ± 4.4											
	Comorbidities														
	Hypertension (N=74)			56.9%											
	Cardiovascular disease (N=39)			30.0%											
	Dyslipidemia (N=46)			46.9%											
	Hypercholesterolemia (N=33)			25.4%											
	Type 2 diabetes mellitus (N=21)			16.2%											
	B) Groups of Patients and Controls Classified According to Morbidities														
	CVD (N=39) vs C (N=91)			**HT (N=74) vs C (N=56)**			**DL (N=46) vs C (N=84)**			**HC (N=33) vs C (N=97)**			**T2DM (N=21) vs C (N=109)**		
	CVD	**C No-CVD**	**p-value**	**HT**	**C No-HT**	**p-value**	**DL**	**C No-DL**	**p-value**	**HC**	**C No-HC**	**p-value**	**T2DM**	**C No-T2DM**	**p-value**
Age (mean± SD)	75.9±6.5	73.7±7.2	0.107	75.2±6.4	73.2±7.8	0.108	74.1±6.1	74.5±7.6	0.770	73.9±6.2	74.5±7.4	0.715	75.1±6.2	74.2±7.3	0.607
Gender (% females)	41.0%	49.4%	p=0.378 χ^2=0.778	44.6%	50.0%	p=0.541 χ^2=0.374	39.1%	51.29%	p=0.188 χ^2=1.736	39.4%	49.5%	p=0.316 χ^2=1.007	47.6%	46.8%	p=0.944 χ^2=0.005
Education in years (mean ± SD)	10.2±4.8	9.9± 4.3	0.729	9.5± 4.0	10.7±4.8	0.119	10.3±4.5	9.8±4.4	0.556	9.8±4.5	10.1±4.4	0.734	9.2±4.2	10.2±4.5	0.382

Abbreviations: HT, hypertension; CVD, cardiovascular diseases; DL, dyslipidemia; HC, hypercholesterolemia; T2DM, type 2 diabetes mellitus.

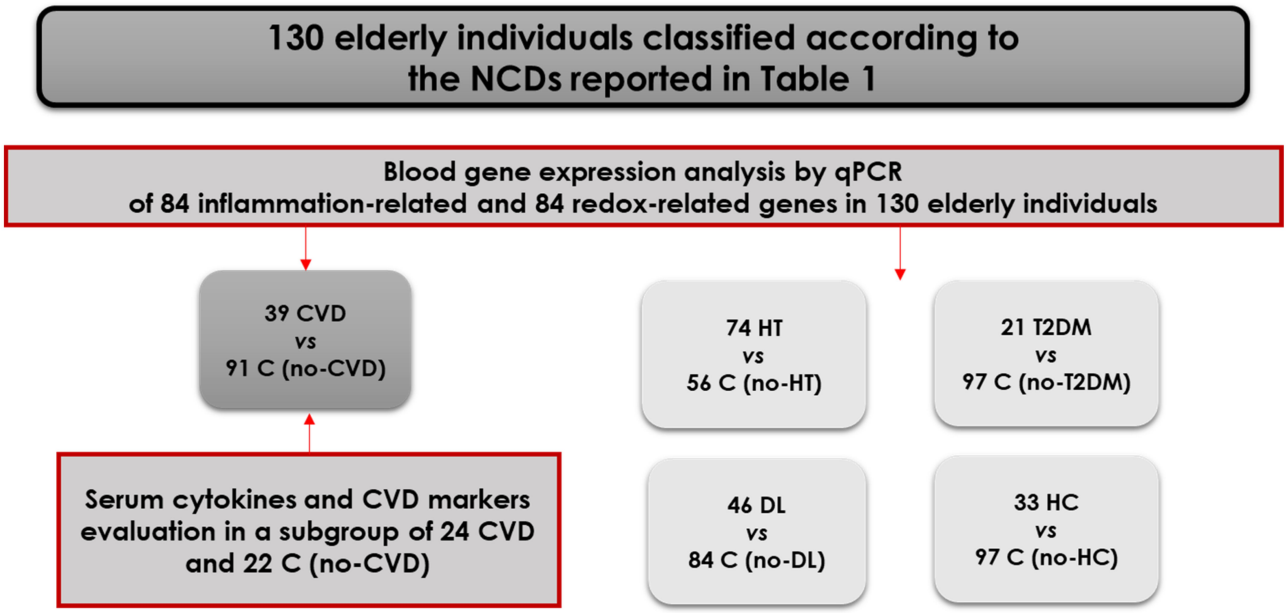

Figure 1 Classification of the 130 recruited elderly patients in case and control groups, according to comorbidities (CVD-cardiovascular diseases, HT-hypertension, DL-dyslipidemia, HC-hypercholesterolemia, T2DM- Type 2 diabetes mellitus). The subjects presenting a type of comorbidity constituted the case group, while all the other subjects in the cohort, not presenting that specific comorbidity, constituted the control group (C).

genes involved in redox responses and of 84 genes related to inflammatory processes (Supplementary Table 1) was evaluated with RT^2 Profiler™ PCR Array Human Oxidative Stress Plus (PAHS-065Y, Qiagen) and RT^2 Profiler™ PCR Array Human NF-κB Signalling Pathway (PAHS-025Z, Qiagen), respectively. The SYBR Green chemistry on an ABI-7500 fast instrument (Applied Biosystems) was applied. The geometric mean of two housekeeping genes (*HPRT1* and *RPLP0*) was used to normalize the expression level of each transcript. These two reference genes were selected among five candidates that are available in each array (ACTB, B2M, GAPDH, HPRT1, and RPLP0), using the RefFinder algorithm (http://leonxie.esy.es/RefFinder/).[20] Gene expression levels were calculated as $2^{-\Delta CT}$ values. Fold Change (FC) in gene expression was calculated as the $2^{-\Delta CT}$ mean values in patients divided by $2^{-\Delta CT}$ mean values in controls. Results are presented as Fold Regulation (FR) as follows: when the FC value was above 1, FR was equal to FC, and results were reported as fold up-regulation; when the FC value was less than 1, FR was expressed as the negative inverse of FC, and results were reported as fold down-regulation.

Protein Multiplexing in Serum

Through protein multiplexing (xMAP array analysis) various cytokines/chemokines/growth factors and CVD markers were assessed in the serum of 24 CVD patients and 22 no-CVD controls, for which serum was available. The xMAP array analysis was performed according to the manufacturer's protocols, and the multiplex data acquisition was done using a Luminex® 200™ system. Serum cytokines, chemokines, and growth factor levels were determined using the following kits from R&D Systems: 1) the Human XL Cytokine Discovery Premixed Kit (R&D Systems), with an analyte-specific bead set addressing CCL11/Eotaxin, CCL2/JE/MCP-1, CCL3/MIP-1alpha, CCL4/MIP-1beta, CD40 ligand/TNFSF5, CX3CL1/Fractalkine, CXCL10/IP10/CRG-2, EGF, G-CSF, IL-13, IL-15, IL-1ra/IL-1F3, IL-3, and IL-7; 2) the Human High Sensitivity Cytokine Base A, addressing TNFα, VEGF, IL-2, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, GM-CSF, and IFNγ. The concentration of the serum CVD markers (CK_MB, TROPONIN1, CXCL6, CXCL16, Endocan1, FABP3, FABP4, PGF, OSM (Supplementary Table 2) was evaluated using the MILLIPLEX MAP Human Cardiovascular Disease (CVD) Magnetic Bead Panel 1 (Merck Millipore). Briefly, the beads were incubated for several hours (depending on the kit) with standards, controls, and samples in a 96-well plate. All further incubations with the Biotin-Antibody Cocktail and with Streptavidin phycoerythrin were performed at room temperature, in the dark, under shaking at 800 rpm. Multiplex data analysis was performed using xPONENT 4.2 software (Millipore) and the calibration curves were generated with a 5-parameter

logistic fit. Duplicate samples were used for all serum specimens, and the mean concentrations per duplicate samples were used for statistical analysis.

Statistical Analysis

For statistical analysis, subjects presenting a particular type of NCD were grouped and were compared with the rest of the subjects from the study cohort which did not have that particular NCD (control group) (Figure 1 and Table 1B). Differences in gene or protein expression levels between groups were evaluated with the Mann–Whitney *U*-test, since the data were not normally distributed (Kolmogorov–Smirnov, $p<0.05$). Only changes in gene expression with absolute FR values >1.8 and p values <0.005 were considered significant in this study. Correlations between gene expression levels were calculated using the Pearson coefficient and were considered significant with a coefficient $r > 0.7$ (positive correlation) and $p \leq 0.001$.

Results

The expression levels of 168 genes involved in inflammation and redox metabolism were analyzed in a cohort of 130 elderly individuals whose demographic and clinical parameters are described in Table 1. The investigated patients presented one or more chronic morbidities that were controlled with polyvalent medication. Analysis was performed according to each of the NCDs registered in the investigated cohort of 130 elderly patients (Table 1A), by comparing the data from subjects presenting a particular morbidity with the data from subjects not having that particular disease (control group) (Table 1B).

We identified a distinctive gene expression profile in CVD patients (N=39) as compared to the corresponding controls, that was not found in the groups of patients presenting primarily hypertension (N=74), dyslipidemia (N=46), including hypercholesterolemia (N=33), or T2DM (N=21) (Supplementary Table 3).

The investigated CVD patients had atherosclerosis (N=21) (carotid atherosclerosis-14, mesenteric artery disease-1, coronary artery disease-6), venous chronic insufficiency (N=2), arrhythmia (N=8) (atrial fibrillation-6, paroxysmal tachycardia-1, sinus node dysfunction-1), angina pectoris (N=5), congestive heart failure (N=2), and cardiomyopathy (N=1). The disease was kept under control by medication as shown by the registered serum concentrations of various CVD biomarkers that were in the normal range (Supplementary Table 2).

The gene expression profile in the blood of CVD patients was characterized by significant down-regulation of several inflammatory genes (Figure 2). These genes are part of the NF-κB system (Figure 2A: *NFKB2, NFKBIA, RELA,* and *RELB*), or participate in signaling pathways interacting with NF-κB (Figure 2B: *AKT1, IRF1,* and *STAT1*), are involved in receptor-mediated signaling through NF-κB (Figure 2C: *CD40, LTA,* and *TRAF2*) and in arachidonic acid metabolism (Figure 2D: *ALOX12* and *PTGS1*). We have further investigated the impact of inflammatory genes down-regulation on the level of 15 pro-inflammatory cytokines/chemokines/growth factors in serum from a sub-group of elderly subjects (24 CVD patients and 22 no-CVD controls). Down-regulation of the inflammation genes identified in 39 CVD patients and 91 controls (Figure 2) was reproduced also in the smaller sub-group for which cytokines were measured (data not shown). All investigated pro-inflammatory factors, excepting IL-1β, were in the normal range. The concentration of IL-1β was decreased to 67% in CVD patients as compared to no-CVD controls (CVD patients: 0.44 pg/mL, no-CVD subjects: 0.75 pg/mL, $p=0.002$). We found no significant correlations ($r>0.5$, $p<0.05$) between the IL-1β decrease and the down-regulated genes in CVD patients.

In parallel to inflammatory genes down-regulation, we observed under-expression of several redox genes in the blood of CVD patients (Figure 3), that are involved in reactive oxygen species (ROS) production (Figure 3A: *DUOX1/2* and *MPO*), glutathione (*GSR*) and thioredoxin (*TXNRD2*) metabolism (Figure 3B), oxidative damage repair (Figure 3C; *HSPA1A* and *MSRA*), and adaptors for cytoskeletal proteins (Figure 3D: *PDLIM1*).

The Pearson correlation analysis performed on the inflammatory and redox genes that were down-regulated in CVD patients indicated significant intra-pathway connections for most of these inflammatory (Figure 4) and redox (Figure 5) genes. However, no significant association was evidenced for the *LTA* gene in the inflammatory pathway (Figure 4B), and for the *GSR* gene in the redox pathway (Figure 5A), suggesting that mechanisms other than those analyzed in our arrays underlie their down-regulation. Intra-pathway correlations were more abundant in the group of CVD patients than in the control group, therefore providing a distinctive CVD signature. For instance, *NFKBIA* was correlated with several genes in the NF-κB-related signaling pathway in the case of CVD patients (Figure 4A), while no significant connections

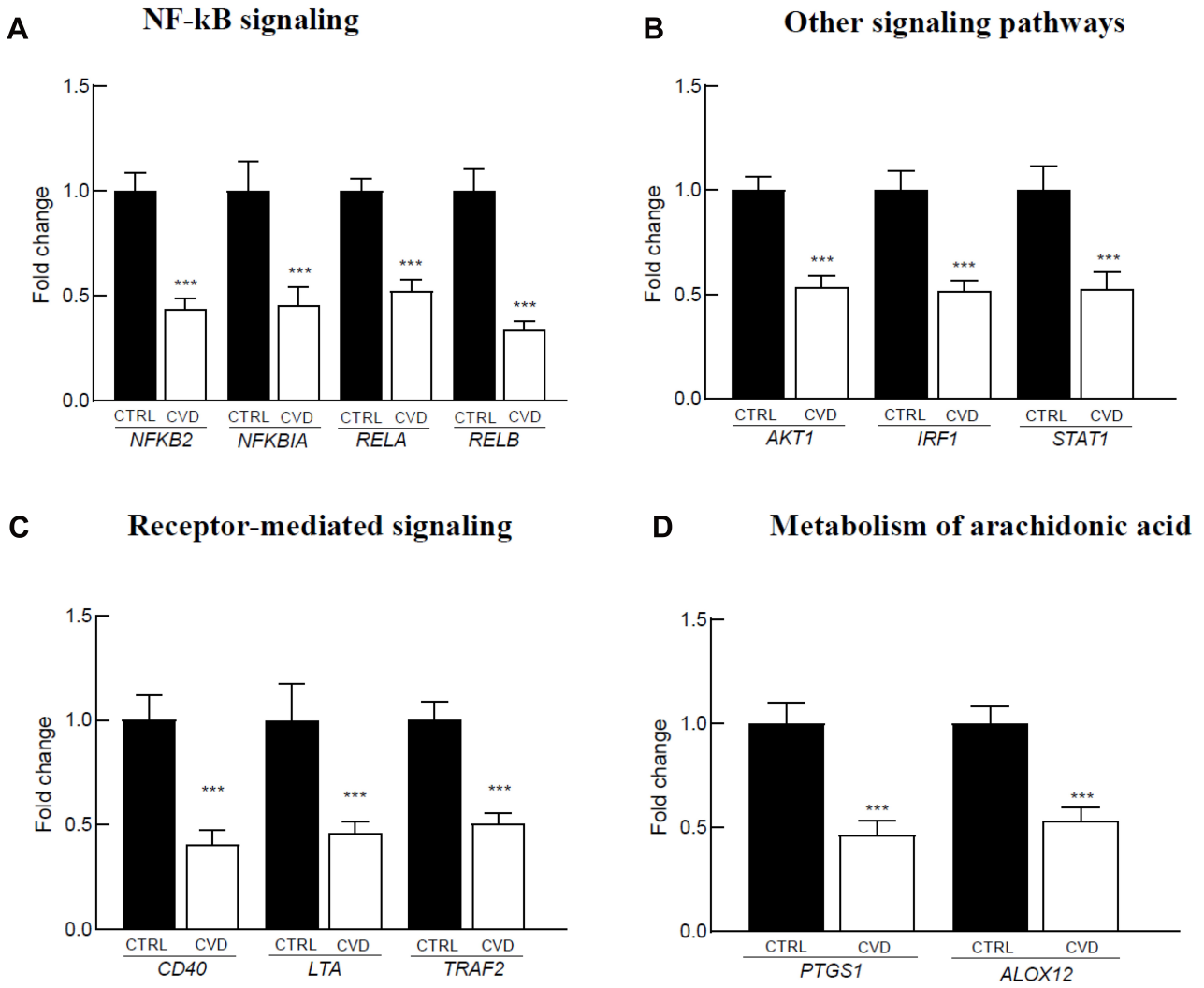

Figure 2 Inflammation-related genes differentially expressed in the blood of 39 CVD patients versus 91 controls. The reported genes are involved in: (**A**) NF-kB signaling, (**B**) Other signaling pathways, (**C**) Receptor-mediated signaling, (**D**) Metabolism of arachidonic acid. Gene expression levels are expressed as mean relative fold of change versus controls and standard error of the mean (SEM). The p-value was calculated using the Mann–Whitney *U*-test (***p<0.001).

were found in the control group (Figure 4B). Likewise, more associations centered on the *RELA* gene were evidenced in CVD patients as compared with the control group (Figure 4A and B). In the case of redox genes, two intra-pathway correlations were registered for the *MPO* gene in CVD patients (Figure 5A), but no significant connections were observed in controls (Figure 5B).

When analyzing the inter-pathway correlations, multiple associations between inflammatory and redox genes were detected in the group of CVD patients (Figure 6A), with the inflammatory *NFKB2, RELA, TRAF2, PTGS1,* and *ALOX12* genes showing the highest number of inter-pathway associations. Regarding redox genes, *TXNRD2, HSPA1A, MSRA,* and *PDLIM1* showed multiple connections with inflammatory genes in CVD patients (Figure 6A). Comparing CVD patients with controls, we found far more inter-pathway correlations in the CVD group (Figure 6A and B). For instance, no correlations of the *NFKB2, RELA, IRF1, AKT1, STAT1, CD40, LTA,* and *TRAF2* genes with redox genes were detected in controls, while multiple connections were evidenced in CVD patients. These results provide a particular transcriptional signature of inflammatory and redox genes identified in the whole blood of CVD patients.

Discussion

The pathway-focused qRT-PCR analysis in whole blood indicates that the investigated CVD patients, with disease kept under control by medication, are characterized by abnormally low expression levels of several inflammatory and redox genes which generally show good intra- and

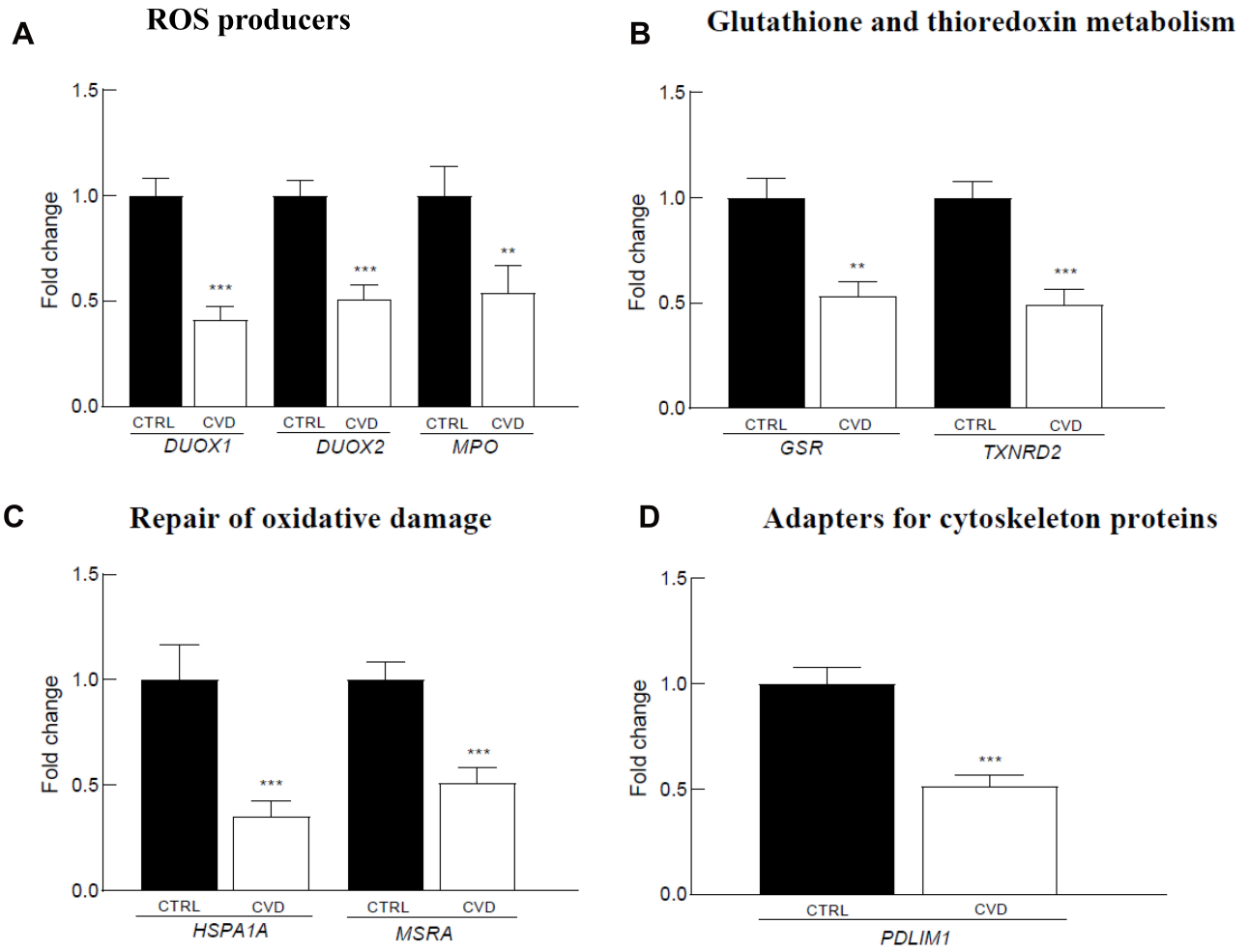

Figure 3 Redox-related genes differentially expressed in the blood of 39 CVD patients versus 91 controls. The reported genes are involved in: (A) ROS producers, (B) Glutathione and thioredoxin metabolism, (C) Repair of oxidative demage, D. Adapters for cytoskeleton proteins. Gene expression levels are expressed as mean relative fold of change versus control and standard error of the mean (SEM). The p-value was calculated using the Mann–Whitney *U*-test (**p<0.01; ***p<0.001).

inter-pathway correlations. Further functional proteomics studies will be required to determine if the observed gene down-regulation correlates with altered inflammatory or redox responses, but we will briefly discuss below their potential implications.

With regard to inflammation, several of the down-regulated genes in CVD patients are involved in the canonical or non-canonical NF-κB pathways (Figure 7). In the canonical NF-κB pathway, we found under-expression of the *RELA* and *NFKBIA* genes, encoding the transcription factor p65-NF-κB and its cognate repressor IκBα, respectively. This apparent contradiction is consistent with the fact that *NFKBIA* contains several κB-responsive elements that participate in a feedback control of the canonical pathway, and therefore the depletion of p65-NF-κB will necessarily lead to the diminution of its repressor.[21–23] The canonical NF-κB pathway is partly responsible for the persistent low-grade inflammation that characterizes most chronic diseases, including CVD,[13,24] and is also pivotal for mounting efficient immune responses against pathogens.[25–27]

The under-expression of genes involved in the non-canonical NF-κB pathway addresses the core genes *NFKB2* and *RELB*, whose products lead to the formation of the transcriptionally active p52/RelB heterodimer. Moreover, the scaffold-encoding gene *TRAF2*, as well as the *CD40* and *LTA* genes encoding members of the tumor necrosis receptor family was also under-expressed. Accordingly, the immune functions governed by this pathway are expected to be impaired in CVD patients, ie, survival and maturation of B lymphocytes, differentiation of dendritic cells into competent antigen presenting cells, and cellular responses against some RNA viruses.[28,29] Altogether, the observed gene under-expression within

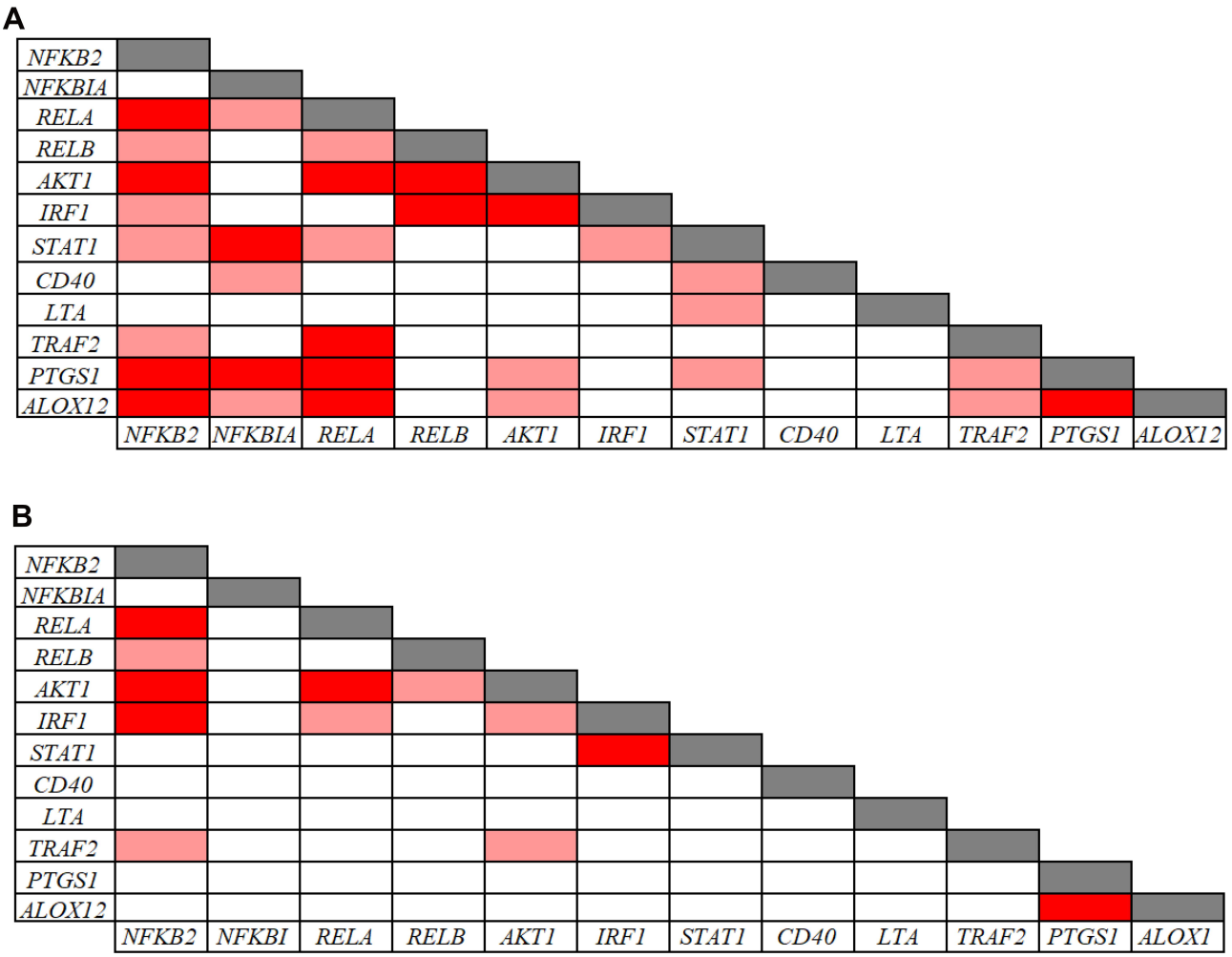

Figure 4 Pearson correlations among inflammation-related gene expression levels in 39 CVD patients (**A**) and in 91 controls (**B**). Correlations with p-values < 0.001 and r values > 0.7 (light red: 0.7 < r < 0.8; dark red: r ≥ 0.8) are presented.

the NF-κB activation pathways might result to some extent in immune suppression.

The down-regulation of the NF-κB pathways in CVD patients was accompanied by the under-expression of several other genes that are under the NF-κB control and/or whose products impinge on the NF-κB system. This was the case of the *AKT1* gene.[30,31] Moreover, the *IRF1* and *STAT1* genes, that shape interferon (IFN)-mediated immune responses against viruses and bacteria, were also under-expressed in CVD patients. *IRF1*, whose transcription is under NF-κB control, encodes a transcriptional regulator of IFN-inducible genes, and *STAT1* participates in an amplification loop of IFN.[32,33]

The suppression of inflammatory pathways in CVD patients is also sustained by the under-expression of genes that are involved in the oxidative metabolism of arachidonic acid. This was the case of the *ALOX12* gene, encoding human platelet-type arachidonate 12-lipoxygenase, and the *PTGS1* gene encoding the constitutive form of cyclooxygenase (COX-1) that catalyzes the formation of cyclic prostanoids with various effects on platelet activation and aggregation.[34–39] In CVD patients, down-regulation of these genes is probably due to the anti-thrombotic medication.[40,41] In addition, *PTGS1* under-expression may affect the priming of the innate immune system by increasing phagocytosis and ROS-mediated killing of bacteria, while *ALOX12* under-expression can impair physiologic inflammatory responses.[42,43]

Concerning the redox metabolism, several related genes were found under-expressed in CVD patients. These include genes involved in ROS production (*DUOX1/2* and *MPO*), antioxidant response (*GSR* and *TNXRD1*), repair of oxidative damage (*HSPA1A* and *MSRA*), and regulatory adapter proteins (*PDLIM1*). The

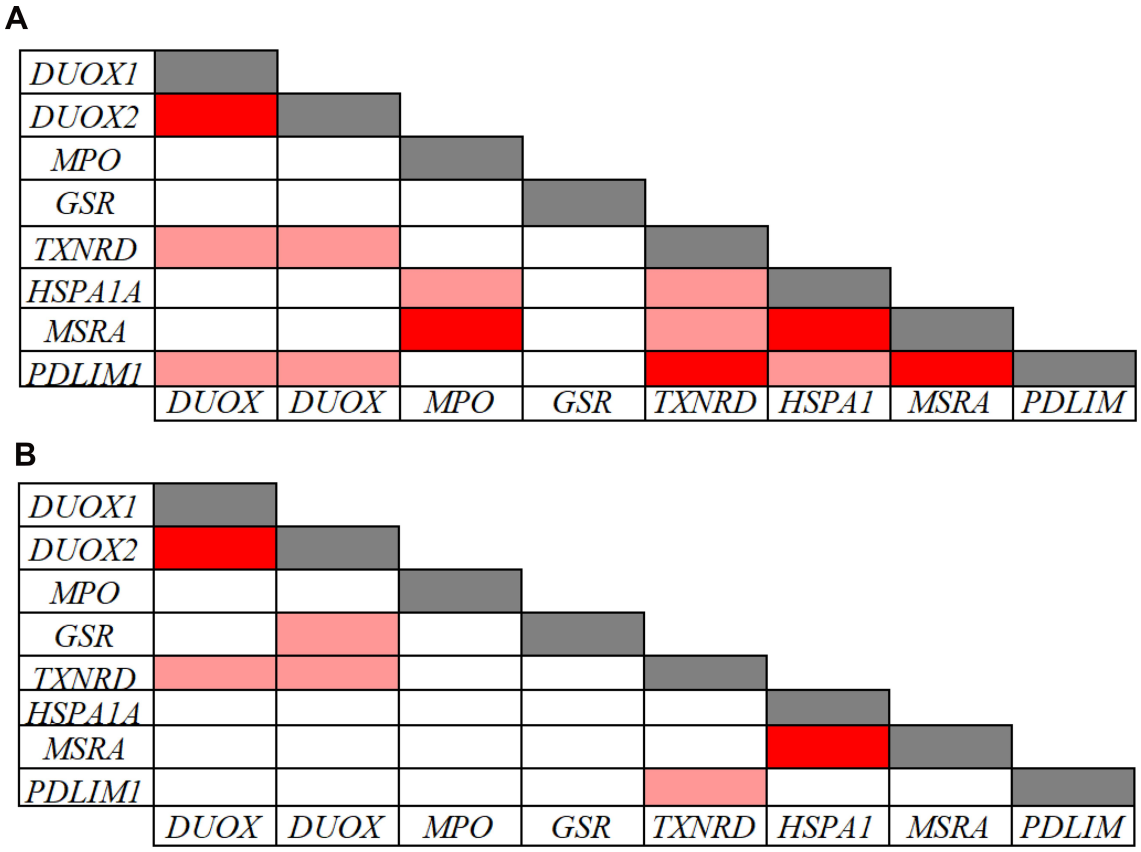

Figure 5 Pearson correlations among redox related-gene expression levels in 39 CVD patients (**A**) and in 91 controls (**B**). Correlations with p-values < 0.001 and r values > 0.7 (light red: 0.7 < r < 0.8; dark red: r ≥ 0.8) are presented.

down-regulation of these genes may have consequences on redox signaling as well as on the oxidative antimicrobial defense. For instance, the under-expression of the *MPO* gene may decrease the ability of blood cells to kill non-specifically invading pathogens through the generated hypochlorous acid.[44] Increased MPO levels are also associated with CVD and with a higher mortality risk, while MPO-deficiency was shown to decrease the susceptibility to neutrophil-mediated vascular dysfunction.[45,46]

Down-regulation of the genes encoding NADPH-oxidases, such as *DUOX1* and *DUOX2*, was reported in the blood of CVD patients.[47,48] Although *DUOX1/2* expression is high in the thyroid gland, these genes are also induced by pathogens and inflammatory cytokines in immune cells through various receptors in connection with NF-κB signaling.[49–52] Therefore, the observed gene under-expression in CVD patients is consistent with the reduction in the levels of the core NF-κB genes and might be related to disease mechanisms and response to therapy, as well as with an impaired antimicrobial defense.[53]

CVD patients exhibited under-expression of the antioxidant genes *GSR* and *TXNRD2* encoding reductases that regenerate the reduced forms of glutathione and thioredoxin, respectively, hence providing antioxidant protection in CVD.[54,55] Other genes identified in the present study are involved in repairing oxidatively damaged, misfolded, and aggregated proteins, namely *HSPA1A* and MSRA.[56–59] Especially *MSRA* is essential during innate immune responses against intracellular microbes for repairing the oxidative damages inflicted to the host's proteins by the innate immune response developed against the invading pathogens.[60,61]

Significant intra-pathway correlations were highlighted in the group of CVD patients for some of the under-expressed inflammatory genes, indicating that the observed changes are most likely integrated in the same signaling networks. In turn, the registered correlations between redox genes seem to indicate only co-occurrence within a disease- or therapy-characteristic pattern and do not appear to reflect functional inter-connection. Some degree of inter-pathway correlations between inflammatory and redox genes was expected, considering that NF-

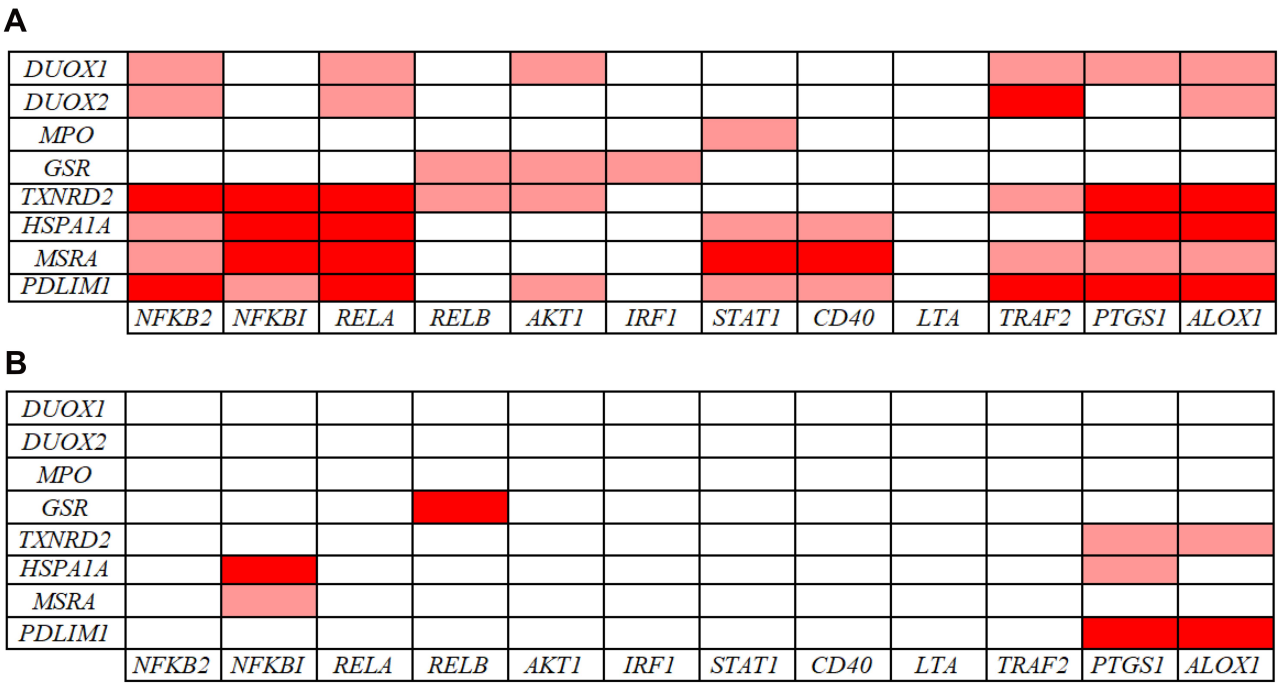

Figure 6 Pearson correlations among inflammation- and redox-related gene expression levels in 39 CVD patients (**A**) and in 91 controls (**B**). Correlations with p-values < 0.001 and r values > 0.7 (light red: 0.7 < r < 0.8; dark red: r ≥ 0.8) are presented.

κB activation is under redox control and that NF-κB controls the expression of several antioxidant genes.[62,63] Nevertheless, part of the observed correlations between inflammatory and redox genes might derive from independent processes that are either disease-specific or triggered by the medication.

The present study suggests that under-expression of critical inflammation and redox genes in the blood of CVD patients underlies their good clinical evolution under disease-specific medication. Additionally, the normal levels of CVD markers and pro-inflammatory factors in serum, observed in the smaller sub-group of 24 CVD patients versus 22 no-CVD controls, indicated that inflammation seems to be kept under control in these patients, and may account for the favorable disease outcome. Moreover, the down-regulation of IL-1β levels in serum is further sustaining this conclusion. In turn, due to the correlated down-regulation of critical inflammatory and redox genes in blood, the immune competence of CVD patients might be compromised, potentially leading to defective antimicrobial responses. Nevertheless, it is likely that the registered relatively low under-expression of several genes involved in the NF-κB and in redox networks might be insufficient to determine a clinically relevant immune suppression leading to recurrent infections.

Considering that CVD is characterized by low-grade inflammation and oxidative stress, the down-regulation of critical inflammatory and redox genes found in the present study most likely reflects the effect of disease-specific polyvalent medication, comprising angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists, beta-adrenergic blocking agents, anticoagulant and antiplatelet drugs, diuretics, and statins (Supplementary Figure 1). Some of these drugs exert antioxidant effects that greatly contribute to limit disease progression, therefore giving a new valence to their intended therapeutic use.[64,65] For instance, beta-blockers can limit pathologic oxidative stress and redox signaling both in the heart and in the circulating immune cells of patients with heart failure.[66] ACE inhibitors prevent vascular superoxide production, increase the bioactivity of nitric oxide, and decrease the activity of various transcription factors such as the ROS-dependent activation of NF-κB in vascular inflammation.[67,68] Drug-induced decrease of ROS levels is further impacting on various redox-sensitive signaling pathways and also on gene expression, therefore having an array of consequences broader than those directly related to oxidative stress.[69,70]

Interestingly, even though both CVD and hypertension were kept under control by medication in the investigated cohorts, and several drugs were used in both disease groups, no significant changes in gene expression were

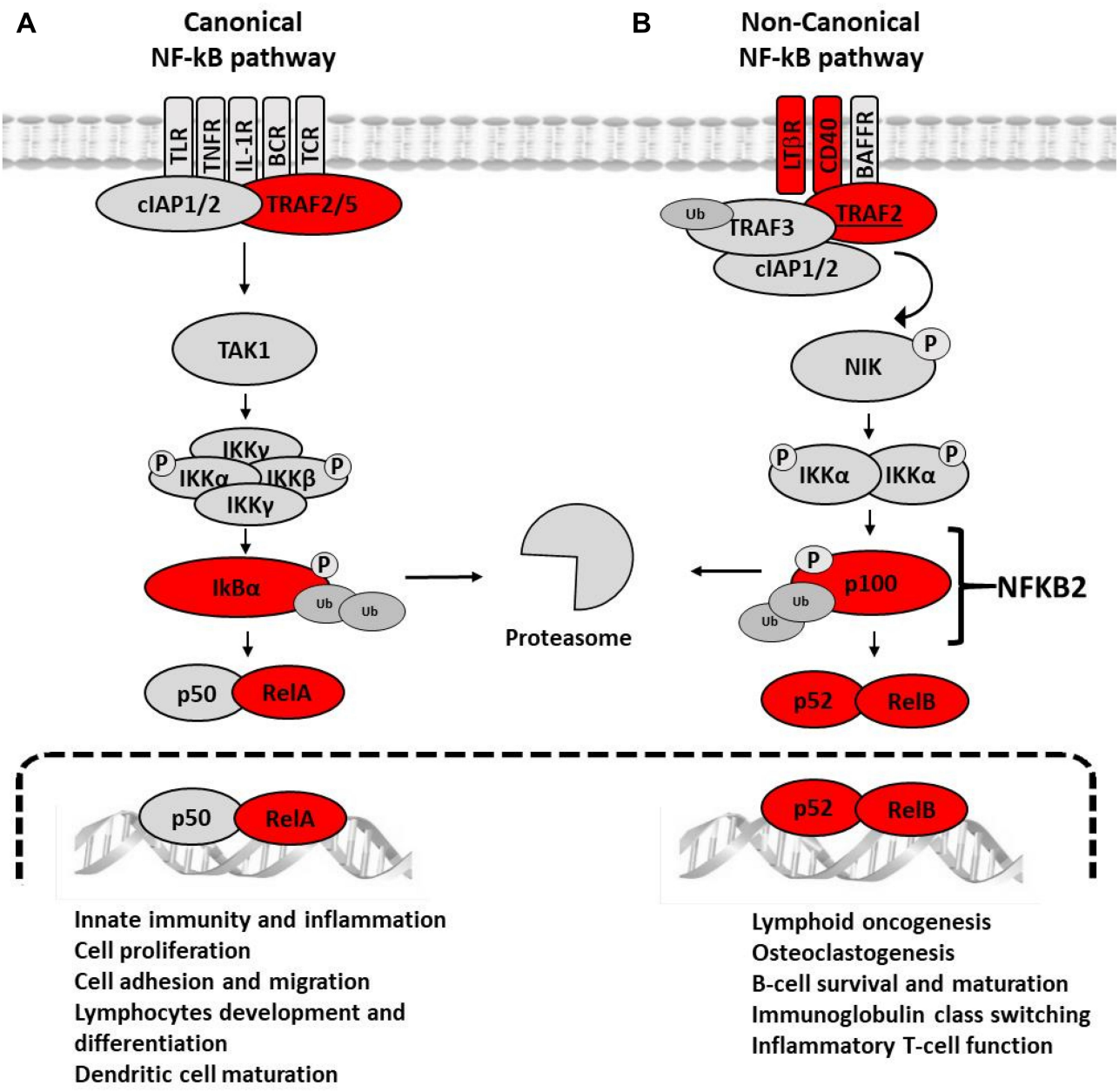

Figure 7 Signaling constituents of the canonical (**A**) and the non-canonical (**B**) NF-κB pathways that are under-expressed in CVD patients. In (**A**), the binding of ligands to particular receptors leads to the activation of an IKK complex (IKKα and/or IKKβ catalytic subunits and two IKKγ molecules). This complex phosphorylates IκBα leading to degradation by the proteasome and consequent formation of the transcriptionally-active p50-RelA complex. In (**B**) the binding of ligands to particular receptors triggers the phosphorylation and processing of p100 (encoded by NFKB2) into the mature p52 protein and subsequent nuclear translocation of the transcriptionally-active RelB-p52 heterodimer. The products of the genes found differentially expressed the present study in the group of patients with CVD are marked with a red circle.

registered in the all-cause hypertension group. This might be due to a distinctive profile of pathological mechanisms in patients with atherosclerosis which dominate in the CVD group. This is possibly resulting also in distinctive or more pronounced effects of medication.

Limitations of the Study

The small number of subjects in each disease group did not allow to stratify patients according to disease sub-categories/stage or therapeutic regimens comprising various combinations of drugs. Based on the results obtained in the present study, broader and more disease- and therapy-focused investigations should be performed for clarifying whether the reported gene expression changes are responsible, at least partly, for keeping disease under control in the context of disease variants and polyvalent medication.

The present study provides data on gene expression changes in whole blood, that might have systemic

consequences that have to be comprehensively investigated through a follow-up of the CVD patients. The functional impact of the registered gene expression changes was not thoroughly assessed in this study, except for an analysis of some cardiovascular markers and pro-inflammatory factors in serum. Therefore, further investigations will be required to determine if the observed gene down-regulations result in decreased levels of the proteins encoded by the identified genes and if these cellular disturbances lead to clinically relevant alterations of inflammatory or redox responses.

Conclusions

The present study highlights the down-regulation of several genes involved in NF-kB-mediated inflammation and redox metabolism in the blood of elderly CVD patients with disease kept under control by polyvalent medication. The study strongly suggests that this specific panel of under-expressed inflammation- and redox-related genes can be used for non-invasively monitoring disease progression and patient engagement to medication in CVD.

Abbreviations

ACTB, Actin Beta; *AKT1*, AKT Serine/Threonine Kinase 1; *ALOX12*, Arachidonate 12-Lipoxygenase, 12S Type; *B2M*, Beta-2-Microglobulin; *CD40*, CD40 Molecule; *DUOX1*, Dual Oxidase 1; *DUOX2*, Dual Oxidase 2; *GAPDH*, Glyceraldehyde-3-Phosphate Dehydrogenase; *GSR*, Glutathione-Disulfide Reductase; *HPRT1*, Hypoxanthine Phosphoribosyltransferase 1; *HSPA1A*, Heat Shock Protein Family A (Hsp70) Member 1A; *IRF1*, Interferon Regulatory Factor 1, *LTA*, Lymphotoxin Alpha; *MPO*, Myeloperoxidase; *MSRA*, Methionine Sulfoxide Reductase A; *NFKB2*, Nuclear Factor Kappa B Subunit 2; *NFKBIA*, NFKB Inhibitor Alpha; *PDLIM1*, PDZ and LIM Domain 1, *PTGS1*, Prostaglandin-Endoperoxide Synthase 1; *RELA*, RELA Proto-Oncogene, NF-KB Subunit; *RELB*, RELB Proto-Oncogene, NF-KB Subunit; *RPLP0*, Ribosomal Protein Lateral Stalk Subunit P0; *STAT1*, Signal Transducer And Activator Of Transcription 1, *TRAF2*, TNF Receptor Associated Factor 2; *TXNRD2*, Thioredoxin Reductase 2.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there is no financial interest or other conflict of interest regarding the publication of this paper.

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