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MicroRNA expression biomarkers of chronic venous disease

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ABSTRACT

Chronic venous disease (CVD) is a common disease caused by hemodynamic disorders of the venous circulation in the lower extremities. The clinical image of this disease is complex and includes such signs as telangiectases, varicose veins, leg edema and skin changes, usually accompanied with ache, pain, tightness, heaviness, swelling and muscle cramps of legs. Venous ulcers develop in the advanced stages of the disease and lead to significant impairment of patient abilities and reduction of the quality of life. CVD is diagnosed based on physical and image examinations, and main treatment options include compression therapy, invasive treatments like endovenous ablation and foam sclerotherapy, as well as pharmacotherapy. Currently, there is no biochemical and molecular biomarkers utilized in diagnosis or treatment of CVD. With regard to this situation, one of the most investigated fields for identification of disease biomarkers is microRNA (miRNA). These constitute a pool of small, non-coding RNAs that play crucial roles in maintaining cellular homeostasis through posttranscriptional regulation of genes expression. Dysregulations of miRNA expression profiles have been found in patients with various diseases, and this situation provides information about potential miRNA signatures involved in pathophysiology. In this review, the studies focused on investigations of miRNA expression patterns in patients with CVD were collected. The performed literature analysis provides contemporary knowledge in the field of miRNA-dependent mechanisms involved in the etiopathogenesis of CVD and shows gaps that need to be filled in further studies.

INTRODUCTION

Pathophysiology, epidemiology and management of chronic venous disease

Chronic venous disease (CVD) is under-recognized in global health-care. This situation is aggravated by its high prevalence and negative socio-economic impact. CVD encompasses a pool of chronic hemodynamic disorders of lower extremities venous circulation, including venous valvular incompetence, venous hypertension, venous reflux and muscle pump dysfunctions [1-3]. Pathological mechanisms involved in CVD development include changes in shear stress and endothelial integrity caused by environmental and genetic factors, leading to enhanced expression of adhesion molecules and leukocyte-endothelial activation. Increased inflammatory response, exacerbated by production of cytokines and chemokines, as well as activation of matrix metalloproteinases, constitute the main cause of venous wall and valves damage. As a consequence of disease progression, further signs, including venous dilation, alterations in the microcirculation, skin changes and venous leg ulcer, could develop [4].

The set of clinical signs associated with CVD has been organized from C0 to C6 classes according to the CEAP (Clinical, Etiology, Anatomic, Pathophysiology) classification (Tab. 1) [1,5-7]. In medical nomenclature, the term "chronic venous insufficiency" (CVI) was introduced to describe the occurrence of more advanced venous disorders belong to C3-C6 classes of CEAP classification [8].

As briefly mentioned, CVD is a vascular disease characterized by very high prevalence. The report from The Vein Consult Program showed that worldwide prevalence of CVD among general clinic outpatients is around 60% [9,10]. Depending on the study, varicose veins, which are one of the early symptoms of this disease, affect 2-56% of all men and 1-60% of all women [11]. In a large epidemiological study conducted in 2003 in Poland, signs of CVD were reported in 47% of all women and 37% of all men [12]. In a subsequent study conducted in Poland involving 13.393 participants, initial symptoms of CVD were observed in 56.1% of all subjects [13]. The adverse socio-economic impact of CVD

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Table 1. Clinical, Etiological, Anatomical and Pathophysiological (CEAP) classification of CVD [7]

Clinical classification (Complemented by "a" for asymptomatic presentation and „s" for symptomatic presentation. Symptomatic presentation includes occurrence of such symptoms as ache, pain, tightness, swelling, burning, skin irritation, sensations of throbbing or heaviness, muscle cramps and restless leg)	
C0	No visible or palpable signs of venous disease
C1	Telangiectases or reticular veins
C2	Varicose veins
C2r	Recurrent varicose veins
C3	Oedema
C4	Changes in skin and subcutaneous tissue: Class 4a – Pigmentation and/or eczema Class 4b – Lipodermatosclerosis and/or atrophie blanche Class 4c – Corona phlebactica
C5	Healed venous ulcer
C6	Active venous ulcer
C6r	Recurrent active venous ulcer
Etiological classification	
Ec	Congenital (eg. Klippel-Trenaunay syndrome, Parkes-Weber syndrome)
Ep	Primary
Es	Secondary
Esi	Secondary – intravenous (eg. deep venous thrombosis)
Ese	Secondary – extravenous (eg. central venous hypertension)
En	No venous etiology identified
Anatomical Classification	
As	Superficial veins
Ad	Deep veins
Ap	Perforating veins
An	No venous location identified
Pathophysiological classification	
Pr	Reflux
Po	Obstruction
Pr,o	Reflux and obstruction
Pn	No venous pathophysiology identified

is particularly determined by the occurrence of venous ulcers, which significantly reduces the daily activity of patients and their quality of life [1,14]. The most important risk factors for CVD are common and include age, obesity, smoking, low physical activity, chronic and prolonged standing or sitting, pregnancy and a family history [1,9,15].

Methods used for diagnosis of CVD include medical history analysis and physical examination supported by imaging tests. Visual assessment and palpation of the lower extremities is complemented by image examination using e.g. ultrasound venous duplex method. Other non-invasive diagnostic tests are: air plethysmography, computed tomography, magnetic resonance imaging, photoplethysmography, strain gauge plethysmography and foot volumetry. Diagnostic invasive tests include contrast venography, intravascular ultrasound examination and intravascular measurement of venous pressure [1,16-18].

The main aims of therapeutic procedures applied for CVD treatment are to reduce symptoms, halt disease progression, and prevent complications. The recommended therapeutic method for all clinical CEAP degrees is compression therapy performed using specialized garments. The pharmacotherapy of CVD includes using either natural phlebotropic substances (diosmin, rutin derivatives, esculin, escin) or synthetic drugs (tribenozide, calcium dobesylate). In advanced stages of CVD, more invasive methods can be applied, such as foam sclerotherapy, endovascular ablation and surgical intervention [1,6,18,19].

There is still a need, however, to develop new, more efficient methods for the diagnosis and treatment of CVD. Although the clinical signs related to superficial veins are visible and relatively easy to identify, abnormalities affecting deeper localized veins are difficult to detect and require the use of imaging techniques, which demand specialized equipment and highly qualified staff. Furthermore, the effectiveness of currently used treatment methods is not sufficient, especially in the advanced stages of CVD development. Studies focused on the identification of molecular or biochemical biomarkers of CVD could substantially contribute to improving CVD management by providing new targets for CVD diagnosis and treatment. To date, there are no such biomarkers introduced into clinical procedures regarding patients with CVD; however, altered circulatory levels of some promising factors, including estradiol, homocysteine, vascular endothelial growth factor, and miRNAs, were found to be strongly associated with CVD [20]. In the future, the application of methods based on the new targets could result in a higher detection rate, a lower prevalence of complications, reduced treatment costs, and improved quality of life for patients.

Research approach

This paper has the intent of informing readers of the experimental studies focused on comparative miRNA expression analyses utilizing clinical material collected from individuals in various stages of CVD as well as healthy controls (Tab. 2). The purpose of this work is to collect actual knowledge about differentially expressed miRNAs, which were proposed as candidates for biomarkers of CVD. To retrieve articles relevant to this review, PubMed and Google Scholar databases were searched with the following keywords: "miRNA", "microRNA", "expression", "chronic venous disease", "chronic venous insufficiency", "venous ulcer", "biomarker", and their combinations. The abstracts and full text of articles written in English were further screened and the final set of papers was selected by the authors.

MicroRNA – biogenesis and molecular function

Currently, one of the most intensively studied fields of search for markers of cardiovascular diseases is the universe of non-coding RNAs, with special interest in microRNAs (miRNAs) [21-23]. The miRNA pool is comprised of short (18-25 nucleotides), single-stranded and non-coding RNA molecules that exhibit a regulatory function of gene expression. The biogenesis of miRNA starts in the cell nucleus, where miRNA-encoding genes are transcribed by RNA polymerase II, producing primary transcripts of miRNAs (pri-miRNAs). Pri-miRNAs are subsequently digested to miRNA precursors (pre-miRNAs) by RNase III Drosha protein. Pre-miRNAs are exported to the cytoplasm by exportin 5 (XPO5) transporter and processed

Table 2. The summary of studies regarding identification of miRNA expression biomarkers of CVD

Ref.	Cases vs Controls	Material	Method	Differentially expressed miRNAs
[37]	5 males with CVD *vs* 5 male controls	Proximal part of great saphenous vein tissue	Microarray	↑miR-378, miR-378*, miR-376c, miR-202, miR-127-3p, miR-654-3p, miR-376a, miR-196a, miR-34a ↓miR-29b-1*, miR-210, miR-155, miR-338-3p, miR-146b-5p
			RT-PCR validation	↑miR-34a, miR-202 ↓miR-155
[38]	33 patients with varicose veins (varicose vein tissues *vs* normal segments from the same patient)	Veins tissues	RT-PCR	↑miR-202-3p
[39]	8 patients with varicose veins *vs* 8 controls	Veins tissues	Microarray	↑hsa-miR-216a-5p, hsa-miR-136-5p ↓hsa-miR-642a-3p, hsa-miR-718, hsa-miR-4459, hsa-miR-135a-3p, hsa-miR-363-3p
[40]	34 patients with varicose veins *vs* 19 controls	Peripheral blood mononuclear cells	Next Generation Sequencing	↑hsa-miR-122-5p, hsa-miR-3591-3p, hsa-miR-183-5p, hsa-miR-1277-3p, hsa-miR-548d-3p, hsa-miR-34a-5p, hsa-miR-576-3p, hsa-miR-454-3p, hsa-miR-548d-5p, hsa-miR-186-3p, hsa-miR-548aa, hsa-miR-33a-5p, hsa-miR-590-3p, hsa-miR-548t-3p, hsa-miR-1277-5p, hsa-let-7b-3p, hsa-miR-96-5p, hsa-miR-548ac, hsa-miR-19a-3p, hsa-miR-206, hsa-miR-497-3p, hsa-miR-208a-3p ↓hsa-miR-92a-3p, hsa-miR-874-5p, hsa-miR-106b-3p, hsa-miR-181a-2-3p, hsa-miR-128-3p, hsa-miR-769-5p, hsa-miR-30e-3p, hsa-miR-1250-5p, hsa-miR-25-3p
[42]	10 patients with varicose veins *vs* 5 controls	M2-macrophages derived from monocytes	RT-PCR	↓miR-661, miR-1202
[45]	10 patients with venous ulcers *vs* 5 controls	Skin biopsies	RT-PCR	↑miR-16, miR-20a, miR-21, miR-106a, miR-203, miR-130a
[46]	Venous ulcers *vs* normal wounds and skin	Wound-edge epidermal keratinocytes	RT-PCR	↑miR-34a, miR-34c

↑ – upregulated miRNAs, ↓ – downregulated miRNAs

to miRNA:miRNA* duplexes by another RNAse III enzyme: Dicer. One strand (mature miRNA) of the duplex is incorporated into the miRISC protein complex (miRNA associated RNA induced silencing complex) and the other strand typically undergoes degradation. Mature miRNA assembled with miRISC complex can bound to the mRNA strand, either to the 3'UTR, 5'UTR or coding region. Formation of miRNA:mRNA assembly impairs mRNA expression by repression of translation or destabilization and cleavage of mRNA [24-26]. In this way, miRNAs exhibit a pleiotropic effect on protein pool in cells; therefore, even slight dysregulations in miRNA levels may lead to disturbances in physiological processes and development of diseases [27].

Computational databases allow determining genes (both computationally predicted or experimentally validated) targeted by particular miRNAs and indicate related biological processes and signaling pathways. For these reasons, alterations in miRNA expression patterns constitute a subject of intensive research so as to identify markers that can be used in the diagnosis and treatment of human diseases. Many studies demonstrate the critical role of miRNA in pathogenesis of cancer, autoimmunology, infections, atherosclerosis and many other diseases [28-33]. Numerous evidences also show the important role of miRNAs in processes strongly associated with vascular pathology such as angiogenesis, endothelial dysfunction, and vascular inflammation; therefore, dysregulations in miRNA expression profiles could point to promising candidates for biomarkers of CVD [34-36].

MicroRNAs – promising candidates for CVD biomarkers

The following is a listing and description of studies involving the search for miRNA markers of CVD.

Cui *et al*. performed genome-wide miRNA profiling using the microarray method in great saphenous vein samples collected from 5 male patients with CVD (C4 class of CEAP) and 5 control males [37]. Differential expression analysis showed 14 miRNAs with a statistically significant change in expression in the CVD group when compared to controls. Among selected miRNAs, 9 miRNAs were upregulated (miR-378, miR-378*, miR-376c, miR-202, miR-127-3p, miR-654-3p, miR-376a, miR-196a, and miR-34a) and 5 miRNAs were downregulated (miR-29b-1*, miR-210, miR-155, miR-338-3p, and miR-146b-5p). Dysregulation of three miRNAs (miR-34a, miR-155, and miR-202) were confirmed in the validation step using real-time RT-PCR. Functional analysis of genes predicted as targets of 14 differentially expressed miRNAs demonstrated strong association with apoptosis, regulation of proliferation and actin cytoskeleton organization [37].

Higher expression of miR-202 in varicose vein tissues was confirmed later in the study performed by Huang *et al*. using real-time qPCR [38]. Upregulation of miR-202 was associated with important hallmarks of CVD pathogenesis, among others, increased proliferation and migration of VSMC, production of collagen I and induction of oxidative stress. The proposed mechanism for the generation of such effects is the targeting of the 3'-UTR region of PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α) by miR-202. Hence, this miRNA is a promising target for CVD treatment [38].

In their study, Anwar *et al*. performed integrated metabolic and miRNA expression profiling of varicose and normal vein specimens [39]. In doing so, the authors analyzed aqueous and lipid metabolite extracts of vein tissues using 600 MHz 1H Nuclear Magnetic Resonance spectroscopy and Ultra-Performance Liquid Chromatography Mass Spectrometry. Moreover, a microarray hybridization technique was employed for miRNA expression analysis. The outcome of this work was that increased expression level of hsa-miR-216a-5p and -136-5p, as well

as decreased expression level of hsa-miR-642a-3p, -718, -4459, -135a-3p, and -363-3p were found in varicose veins specimens when compared to control tissues. In addition, expression levels of hsa-miR-642a-3p, -4459, and -135a-3p were highly positively correlated, whereas hsa-miR-136-5p was negatively correlated with inosine levels. The results indicate a potential role of dysregulated miRNAs in such inosine-mediated functions as response for hypoxia, Akt signaling pathway, membrane trafficking, cell survival, growth and proliferation. Furthermore, expression levels of miR-216a-5p showed high positive correlation with a variety of other metabolites, including phosphatidylcholine and phosphatidylinositol (a crucial component of the lipid bilayer).

Beyond the aforementioned, another dysregulated miRNA, hsa-miR-142-5p, showed strong positive correlation with sphingomyelin, as well as negative correlation with creatine levels, suggesting the important role of this miRNA in transmembrane signaling and intracellular vesicle trafficking [39].

Our research group performed integrated, experimental miRNA and gene expression analysis in PBMCs (peripheral blood mononuclear cells) isolated from whole blood samples collected from 34 patients diagnosed with CVD (C2 class of CEAP classification) and 19 healthy controls [40]. The application of high-throughput Next Generation Sequencing method followed by multi-stage statistical analysis (DESeq2 and UVE-PLS methods) allowed us to identify 31 miRNAs and 62 genes as significantly differentially expressed in CVD group when compared to controls. Moreover, additional in silico analysis performed using computational databases disclosed 63 regulatory interactions between identified miRNAs and genes, enabling construction of the regulatory network potentially involved in CVD pathogenesis. Functional analysis of miRNA-regulated genes also revealed numerous associations, including links to cardiovascular diseases and risk factors, tobacco use disorder, developmental processes and RNA metabolism [40].

MMP9 (matrix metallopeptidase 9) is one of the factors responsible for extracellular matrix degradation and venous wall weakness during development of varicose veins [41]. Biranvand *et al*. using real-time PCR, examined expression levels of MMP9 and its predicted modulators: lncRNA-GAS5, lncRNA-HOTAIR, miR-661 and miR-1202 in M2-macrophages differentiated from monocytes collected from patients with varicose veins, and compared these to cells from healthy subjects [42]. According to study results, the expression level of MMP9 was shown to be increased, while expression levels of its modulators were decreased in patients with varicose veins. The outcome of this work suggests that lncRNA-GAS5, lncRNA-HOTAIR, miR-661, and miR-1202 play regulatory roles in MMP9 function during CVD development, and that further studies are required to evaluate their potential diagnostic and therapeutic utility [42].

Circular RNAs (circRNAs) have been established as negative regulators of miRNAs [43]. Zhang *et al*. applied high-throughput sequencing to perform a circular RNAs expression profiling of primary great saphenous vein varicosities in comparison to control tissues [44]. Therein, three out of 232 differentially expressed circRNAs (hsa-circ-0006427, -0089810 and -0005267) were found to have the most significant diagnostic potential. Furthermore, computational analysis of all identified differentially expressed circRNAs revealed top 10 downstream miRNAs: hsa-miR-103a-2-5p, -141-5p, -3692-5p, -4659a-3p, -4659b-3p, -4691-5p, -4778-3p, -6738-3p, -6792-3p, and -6873-3p. The potential role of these miRNAs in CVD development was subsequently explored and the most enriched functional terms were found to be related to catabolic processes, ATP binding, axon guidance, vitamin digestion and absorption, and the NF-kappaB signaling pathway [44].

Two another studies identified differentially expressed miRNAs in patients with venous ulcers – a more advanced stage of CVD. Using quantitative PCR and in situ hybridization, increased expression of miR-16, -20a, -21, -106a, -203, and -130a was found in 10 skin biopsies from venous ulcers, as compared to 5 healthy skin specimens [45]. These miRNAs were predicted to target mRNA of genes involved in wound healing, including EGR3 (early growth response 3, transcriptional factor for genes involved in cell proliferation), VCL (vinculin, the main component of the focal adhesion complex) and LEPR (leptin receptor, a hypoxia-inducible cytokine required for wound healing process). Two out of these miRNAs, miR-21 and miR-130a, were experimentally confirmed to directly target LEPR. Application of these miRNAs mimics on wounds delayed wound healing via a suppressing effect on epithelialization of human skin. Hence, therapeutic targeting of miR-21 and miR-130a emerges as a potential option to accelerate healing of venous ulcers [45].

Wu *et al*. have found two upregulated miRNAs in the wound-edge epidermal keratinocytes of venous ulcers: miR-34a and miR-34c [46]. They then demonstrated that both miRNAs enhance the production of inflammatory chemokines and cytokines, probably through targeting mRNA for LGR4 (leucine-rich repeat containing G protein-coupled receptor 4). Subsequent administration of miR-34a mimic in mice promoted inflammation and caused retardation of wound healing. Application of miR-34a and miR-34c antagomirs may constitute new opportunity in the treatment of venous ulcers [46].

Disturbances in miRNA regulatory function involved in CVD pathology could be a result of genetic variabilities within modulated genes. Jin *et al*. examined the association between rs3917 indel polymorphism in the 3'UTR region of the COL1A2 (collagen type I alpha 2 chain) and the risk of CVI [47]. The authors included 254 CVI cases and 508 controls to the study and brought to light the significantly increased risk of CVI (1.6 fold, $P = 0.008$) in subjects carrying rs3917 polymorphism in COL1A2. Interestingly, this polymorphism is localized in a predicted binding site for miRNA-382 and therefore could impair miRNA-382-mediated suppressing of COL1A2, presumably leading to upregulation of COL1A2 [47]. This effect may explain the previously reported increase in expression of collagen type I in dermal fibroblasts derived from patients with varicose veins; however, further studies are needed to confirm this hypothesis [48].

CONCLUSION

CVD is one of the major health problems worldwide due to its high prevalence, multifactorial character, diverse symptomatology, severe clinical implications and negative socioeconomic impact [1,9]. All these reasons raise the need for research on the understanding of pathological mechanisms in CVD and offer new diagnostic and treatment alternatives. Results of the presented studies confirm the significant role of miRNA-associated regulatory mechanisms in etiology of CVD, and such work points out that altered expression of miRNA could be a promising research area for the search of novel biomarkers with potential clinical utility.

Unfortunately, the efforts that have been made to date are obviously insufficient and further studies are strongly needed in this field. The studies presented in this review largely focused on vein tissue samples and further investigations should employ high-accessible clinical material, such as whole blood, serum, plasma or exosomes, which will facilitate the prospective application of obtained results in clinical practice. Further studies should also be integrated with whole transcriptome expression analysis in order to obtain deeper insight into the relationships between miRNA and regulated genes, and the consequential effects on the pathological processes. Moreover, previous studies included a small number of participants; therefore, further investigations should involve much larger populations characterized in detail, demographically matched and preferably divided into CVD stage-dependent subgroups. The results obtained in such studies may then be more meaningful for elucidation of miRNA-dependent regulation in the pathogenesis of CVD and for the identification of precise and robust miRNA biomarkers with high diagnostic and therapeutic potential.

LIST OF ABBREVIATIONS

ABCA1 – ATP binding cassette subfamily A member 1
CEAP – Clinical, Etiology, Anatomic and Pathophysiology classification of CVD
circRNAs – circular RNAs
COL1A2 – collagen type I alpha 2 chain
CVD – Chronic Venous Disease
CVI – Chronic Venous Insufficiency
EGR3 – early growth response 3
LEPR – leptin receptor
LGR4 – leucine rich repeat containing G protein-coupled receptor 4
MAPK – mitogen-activated protein kinases
miRNA – microRNA
MMP9 – matrix metallopeptidase 9
PBMCs – peripheral blood mononuclear cells
PTEN – phosphatase and tensin homolog
qPCR – quantitative Polymerase Chain Reaction
RT-PCR – Reverse Transcription - Polymerase Chain Reaction
VCL – vinculin
VSMC – vascular smooth muscle cells

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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