



Article

Functional Differences Between Typical and Multinucleated Endothelial Cells Under Low-Density Lipoprotein Exposure

Vadim Cherednichenko ^{1,*} , Diana Kiseleva ^{1,2} , Ulyana Khovantseva ^{1,3} , Denis Breshenkov ¹ ,
Rustam Ziganshin ⁴, Olga Dymova ¹, Tatiana Kirichenko ^{1,3} , Eduard Charchyan ¹ and Alexander M. Markin ^{1,5}

¹ Petrovsky National Research Center of Surgery, 119435 Moscow, Russia

² Department of Biophysics, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia

³ Department of Biology and Genetics, Petrovsky Medical University, 119435 Moscow, Russia

⁴ Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry Russian Academy of Sciences, 117997 Moscow, Russia

⁵ Department of Histology, Petrovsky Medical University, 119435 Moscow, Russia

* Correspondence: cherednichenko_vadim@bk.ru

Abstract

Endothelial cells are key regulators of vascular homeostasis, and their dysfunction plays a central role in the development of atherosclerosis and other cardiovascular diseases. Multinucleated variant endothelial cells (MVECs) have been described in pathological vascular regions; however, their functional properties remain poorly characterized. The aim of the present study was to compare lipid handling, inflammatory activation, barrier-associated features, and secretory profiles of typical endothelial cells (TECs, EA.hy926 line) and MVECs under low-density lipoprotein (LDL) exposure. MVECs were generated by polyethylene glycol-induced fusion of EA.hy926 cells and incubated with LDL under standardized conditions. Intracellular cholesterol accumulation was assessed biochemically, cytokine secretion was quantified by ELISA, gene expression of inflammatory, endothelial, junctional, and vasoactive markers was analyzed by quantitative real-time PCR, and the endothelial secretome was characterized using data-independent acquisition liquid chromatography–tandem mass spectrometry (DIA-LC-MS). MVECs demonstrated enhanced cholesterol accumulation compared with TECs following LDL exposure. At the transcriptional level, MVECs were characterized by elevated basal expression of proinflammatory markers, including IL1B, IL6, and NFkB1, and showed a markedly amplified IL6 and IL8 response to LDL. In parallel, MVECs exhibited reduced expression of genes associated with antioxidant defense (SOD1), barrier integrity (TJP1), and hemostatic function (VWF). Consistent with transcriptional data, mass spectrometry-based secretome analysis revealed decreased secretion of von Willebrand factor (vWF), vascular endothelial growth factor C (VEGFC), and endothelin-1 (EDN1) by MVECs, accompanied by increased secretion of tissue-type plasminogen activator (t-PA). Functional enrichment analysis of secretome-associated proteins highlighted pathways related to extracellular matrix–receptor interaction, focal adhesion, cell adhesion molecules, complement and coagulation cascades, and leukocyte transendothelial migration. In contrast, TECs demonstrated a more pronounced transcriptional response in EDN1, consistent with their role in vascular tone regulation. Immunocytochemical analysis further revealed altered subcellular distribution of the tight junction protein ZO-1 in MVECs, indicating junctional destabilization. Taken together, these results indicate that MVECs represent a distinct endothelial phenotype characterized by enhanced lipid accumulation, sustained proinflammatory activation, altered secretory signaling, and reduced barrier and hemostatic potential. Such features suggest that MVECs may contribute to the maintenance of chronic endothelial dysfunction and vascular inflammation under conditions of lipid overload.



Academic Editors: Junjie Yang and
Xianming Zhang

Received: 3 February 2026

Revised: 2 March 2026

Accepted: 4 March 2026

Published: 6 March 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

Keywords: endothelial cells; multinucleated variant endothelial cells; endothelial dysfunction; low-density lipoproteins

1. Introduction

Endothelial cells (ECs) line the inner surface of blood vessels and play a key role in maintaining vascular homeostasis, regulating permeability, inflammation, and angiogenesis [1]. Chronic damaging factors such as hyperlipidemia, ischemia, and oxidative stress affect endothelial cells and trigger the development of endothelial dysfunction (ED) [2]. ED represents an early stage in the pathogenesis of cardiovascular diseases, including atherosclerosis, hypertension, and coronary heart disease [3]. ED is characterized by reduced bioavailability of nitric oxide, imbalance of vasoactive factors, increased expression of adhesion molecules, and enhanced secretion of proinflammatory cytokines, ultimately leading to impaired barrier function and vascular inflammation.

A special type of EC has been described in the literature. In atherosclerosis, areas with a large number of multinucleated variants of endothelial cells (MVECs) appear in the aorta [4]. Such zones exhibit enhanced phagocytic activity and correlate with foci of cholesterol and leukocyte deposition in the subendothelial space [5]. Similar cells were found in the study of biopsies of transplanted kidneys with chronic antibody-dependent rejection; their endothelial origin has been confirmed by electron microscopy and immunohistochemistry, and their presence is associated with the development of pathological changes in the graft [6].

MVECs are characterized by increased size (up to $\sim 8000 \mu\text{m}^2$ compared with $\sim 800 \mu\text{m}^2$ in typical endothelial cells) and are multinucleated. It is assumed that they are formed through cell fusion as a result of cellular senescence, rather than as a result of mitotic division [7]. Accumulating evidence suggests that MVECs represent a phenotype reflecting chronic endothelial damage and inflammation. Given that ED is closely associated with the activation of proinflammatory cascades and impaired lipid metabolism, studying the molecular features of MVECs may be important for understanding their contribution to the pathogenesis of vascular diseases.

The present study aimed to compare the phenotype and secretory activity of typical endothelial cells and MVECs, using ELISA to evaluate cytokine production (IL-1 β , IL-6, IL-8, TNF- α), as well as quantitative real-time PCR (qPCR) to determine the expression of genes associated with inflammation (*NFKB1* (nuclear factor kappa-light-chain-enhancer of activated B cells), *IL1B* (interleukin 1 β), *IL6* (interleukin 6), *IL8* (interleukin 8)), intercellular contacts (*OCLN* (Occludin), *TJP1* (Tight Junction Protein 1)), endothelial markers and adhesion molecules (*CD31* (PECAM1, Platelet endothelial cell adhesion molecule-1), *CD146* (MCAM, Melanoma cell adhesion molecule), *VWF* (von Willebrand factor)), and regulation of vascular tone and stress response (*ENOS* (Endothelial nitric oxide synthase), *EDN1* (Endothelin-1), *SOD1* (superoxide dismutase 1)). The data obtained can serve as a basis for understanding the role of MVECs in the pathogenesis of vascular diseases and immune responses.

2. Results

2.1. Endothelial Preparations (Prints)

A total of four endothelial samples from thoracic aortic aneurysm tissue were analyzed: two samples from the aneurysmal lesion itself and two from areas distant from the aneurysmal lesion. In the samples obtained from the aneurysmal sites, MVECs were

detected in large numbers, and they were observed in clusters (Figure 1a,b). Whereas in the “normal” aortic sites, MVECs were not detected (Figure 1c,d).

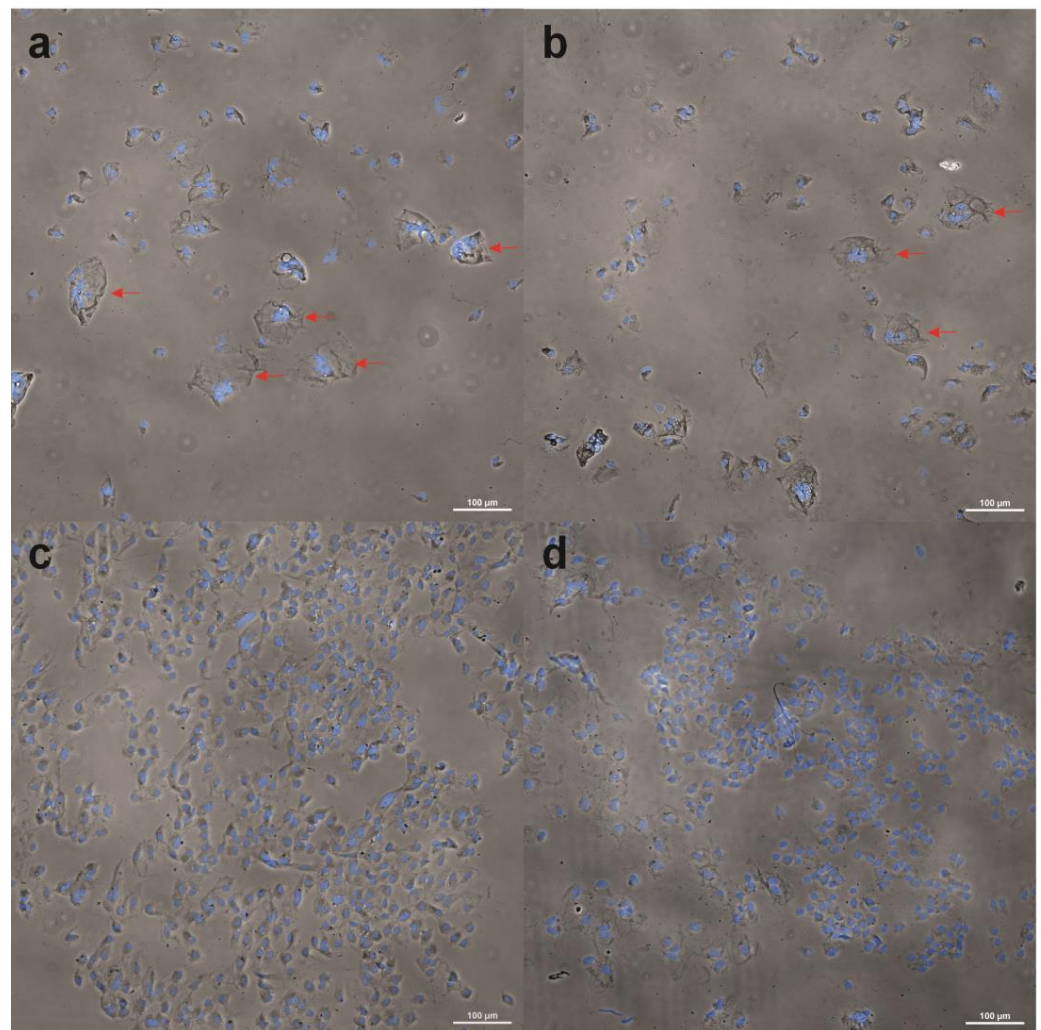


Figure 1. Photographs of endothelial prints obtained in phase contrast mode, additional coloring of DAPI nuclei. (a,b)—the imprint of the endothelium of the aortic area with aneurysmal lesion. The arrows indicate the MVECs. (c,d)—an imprint of the endothelium of the aortic region without aneurysmal lesion. Scale ruler: 100 microns.

2.2. Analysis of Cholesterol Accumulation with Normalization for Total Protein

Incubation with low-density lipoproteins (LDLs) resulted in a significant increase in cholesterol levels in both EC groups: in TECs, cholesterol concentration increased 1.2-fold ($p < 0.001$) compared with the control without LDL; for MVECs, the cholesterol level increased 1.5-fold ($p < 0.001$) relative to the corresponding control group. After incubation with LDL, the cholesterol content in the MVECs was 1.1 times higher ($p < 0.001$) than in TECs. In the absence of LDL, there were no differences between the two cell types (Figure 2).

Cholesterol levels were normalized to total cellular protein. Given the increased MVECs size, normalization to total protein allows for an estimate of lipid accumulation in a heterogeneous cell population, rather than an estimate per individual cell. This approach reflects the contribution of multinucleated cells to the overall lipid load of the endothelium.

These findings indicate that MVECs exhibit enhanced cholesterol accumulation under lipid loading conditions compared with typical endothelial cells.

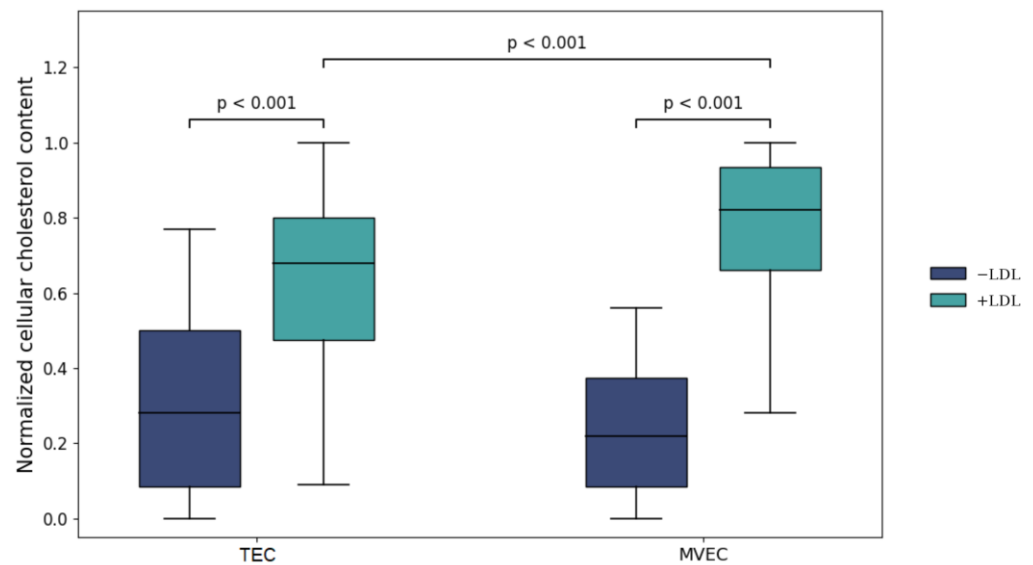


Figure 2. Normalized cellular cholesterol content in TECs and MVECs under control conditions (−LDL) and after LDL treatment (+LDL). Total cellular lipids were extracted using the Folch method. Data are presented as boxplots showing the median, interquartile range, and range.

2.3. ELISA

Incubation of TECs with LDL resulted in a statistically significant increase in IL-6 secretion: median normalized value increased 5.6-fold compared with the control ($p < 0.01$) (Figure 3a). A similar trend was observed for TNF- α , the level of which increased 1.25-fold ($p < 0.05$) (Figure 3d). At the same time, IL-1 β secretion remained stable and did not differ between the control and experimental groups (Figure 3c). IL-8 decreased significantly after LDL exposure, by 4-fold compared with the control ($p < 0.01$) (Figure 3b).

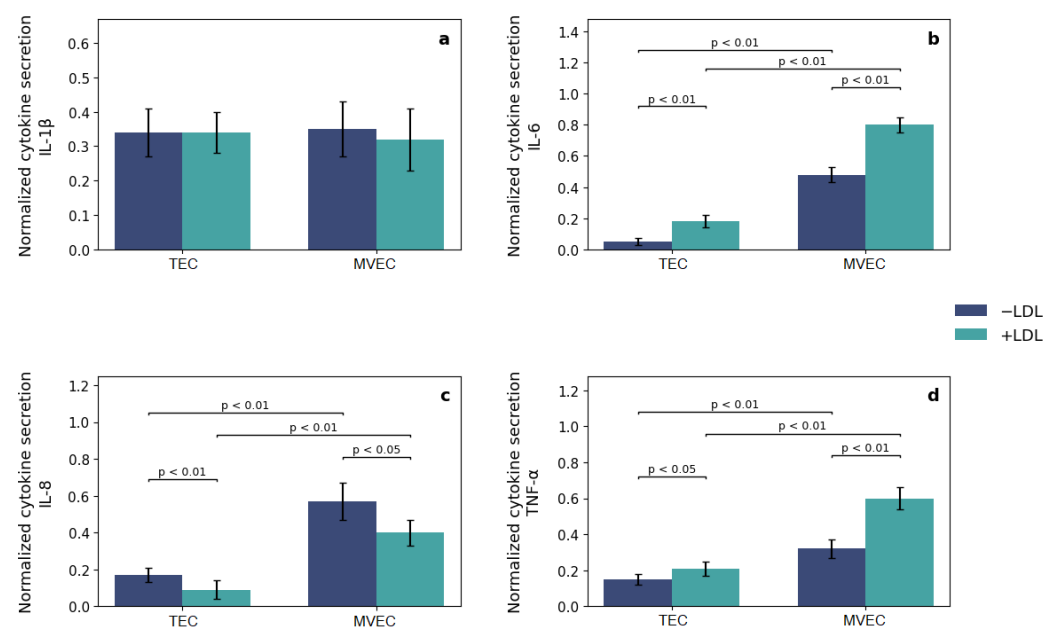


Figure 3. Concentrations of cytokines in the supernatants of TECs and MVECs after 24 h incubation with or without LDL, determined by ELISA: (a) IL-1 β , (b) IL-6, (c) IL-8, (d) TNF- α . Data are presented as normalized values relative to the mean control level of TECs. Each cytokine was measured in at least nine independent biological replicates, each performed in duplicate (technical replicates). Statistical analysis was performed using the Kruskal–Wallis test followed by the Conover multiple comparison test; $p < 0.05$ was considered statistically significant.

MVECs also showed an increase in IL-6 secretion when exposed to LDL: the median value increased by 1.7 times compared with the control ($p < 0.01$) (Figure 3a). TNF- α showed a 2-fold increase ($p < 0.01$) (Figure 3d). IL-1 β remained stable, with no differences observed between groups (Figure 3c). IL-8 decreased 1.2-fold after incubation with LDL ($p < 0.05$) (Figure 3b).

As for the comparison of endothelial cell variants, the MVECs demonstrated significantly higher IL-6 secretion—16.3 times higher than the TECs in the control ($p < 0.01$) (Figure 3a). A similar pattern was found for TNF- α (2.4 times higher, $p < 0.01$) and IL-8 (4.4 times higher, $p < 0.01$) (Figure 3b,d). At the same time, the levels of IL-1 β did not differ statistically between the two cell types (Figure 3c). It should be noted that IL-1 β secretion is tightly regulated at the post-translational level and typically requires inflammasome activation as a second signal. Therefore, unchanged extracellular levels do not necessarily reflect absence of transcriptional regulation.

After exposure to LDL, the differences between cell types persisted. IL-6 remained significantly higher in MVECs—4.8-fold higher than in TECs ($p < 0.01$), TNF- α —3.9 times higher ($p < 0.01$), and IL-8—14.7 times higher ($p < 0.01$) (Figure 3a,b,d). The level of IL-1 β did not differ between TECs and MVECs in experimental conditions (Figure 3c).

Descriptive statistics of cytokine secretion measured by ELISA and effect size analysis are presented in Tables S3 and S4 (Supplementary Materials).

2.4. PCR

2.4.1. Pro-Inflammatory Markers

In TECs, incubation with LDL resulted in a significant increase in *IL8* expression, 1.3-fold higher compared with the control without LDL ($p < 0.001$). In MVECs, the addition of LDL caused a much stronger response: *IL8* expression increased 6.5-fold ($p < 0.001$). At baseline, *IL8* expression in MVECs was 1.9-fold lower than in TECs ($p < 0.001$), whereas after incubation with LDL, *IL8* expression in MVECs exceeded that of TECs by 2.3-fold ($p < 0.001$) (Figure 4g).

In TECs, incubation with LDL led to a significant increase in *IL6* expression, 1.9-fold higher compared with the control group ($p < 0.01$). In MVECs, the response was markedly stronger: *IL6* expression increased 12.3-fold ($p < 0.001$). Basal *IL6* expression in MVECs was 2.7-fold higher than in TECs ($p < 0.001$), and after LDL exposure, this difference increased to more than 27-fold ($p < 0.001$) (Figure 4f).

In TECs, LDL exposure was accompanied by a threefold increase in *IL1B* expression compared with control cells ($p < 0.001$). In MVECs, the response to LDL was less pronounced, with *IL1B* expression increasing only 1.3-fold ($p < 0.001$). However, basal *IL1B* expression in MVECs was significantly higher, 37-fold greater than in TECs ($p < 0.001$). After incubation with LDL, *IL1B* expression in MVECs exceeded that of TECs by 7.7-fold ($p < 0.001$) (Figure 4c).

For *NFKB1*, a statistically significant difference was observed only at the basal level: expression in MVECs was 1.4-fold higher ($p < 0.05$) than in TECs (Figure 4h).

Descriptive statistics of relative gene expression measured by qPCR and effect size analysis are presented in Tables S5 and S6 (Supplementary Materials).

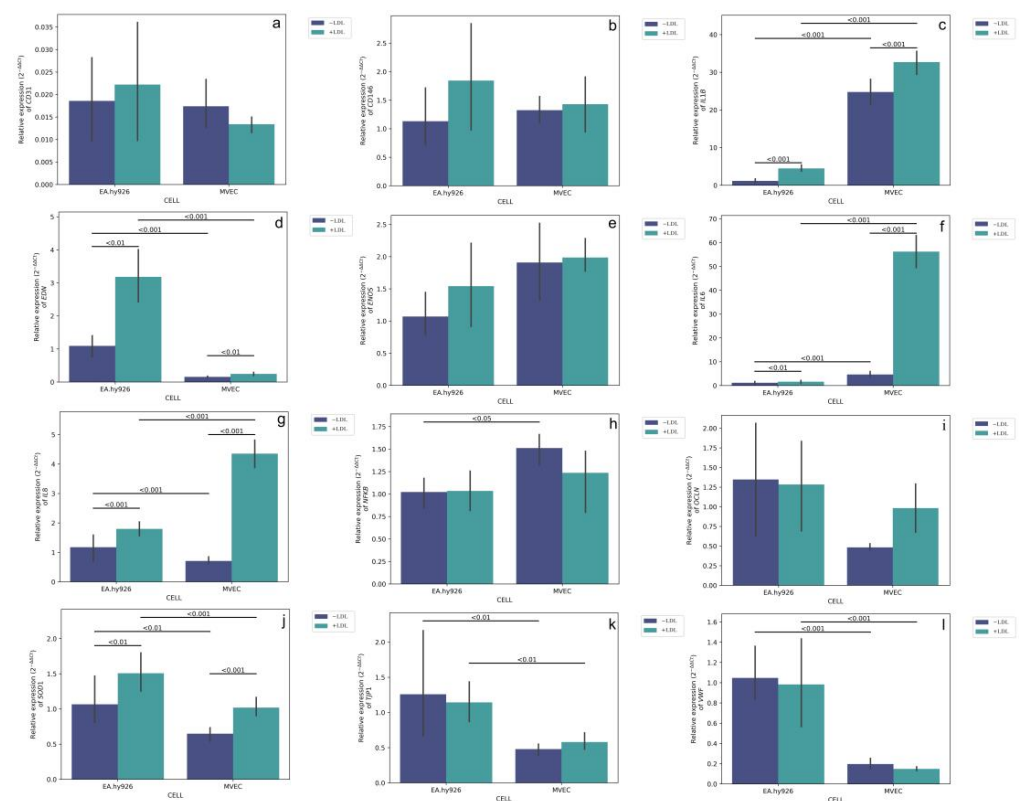


Figure 4. Relative expression of selected genes in TECs and MVECs after 24 h incubation with or without LDL, determined by quantitative real-time PCR: (a) *CD31*, (b) *CD146*, (c) *IL1B*, (d) *EDN1*, (e) *ENOS*, (f) *IL6*, (g) *IL8*, (h) *NFKB1*, (i) *OCLN*, (j) *SOD1*, (k) *TJP1*, (l) *VWF*. Gene expression was normalized to the housekeeping gene *ACTB*, and relative expression levels were calculated using the $\Delta\Delta C_t$ method. Normalization was performed relative to the mean control level of TECs. Each condition was tested in at least six independent biological replicates, each performed in duplicate (technical replicates). Statistical analysis was performed using the Kruskal–Wallis test followed by the Conover multiple comparison test; $p < 0.05$ was considered significant.

2.4.2. Endothelial Markers and Adhesion

CD31 (*PECAM1*) transcription analysis showed comparable expression levels in both TECs and MVECs under conditions with or without LDL. No significant differences were observed between the groups (Figure 4a).

Similarly, no statistically significant differences were detected for *CD146* (*MCAM*). Expression levels remained stable in both TECs and MVECs regardless of LDL exposure (Figure 4b).

LDL exposure did not lead to significant changes in *VWF* expression in either TECs or MVECs. However, comparative analysis revealed marked intergroup differences: the basal level of *VWF* expression in MVECs was 5.2-fold lower than in TECs ($p < 0.001$). After incubation with LDL, this trend persisted, with *VWF* expression in MVECs remaining 6.5-fold lower than in TECs ($p < 0.001$) (Figure 4l).

2.4.3. Markers of Intercellular Junctions and Barrier Function

Incubation with LDL did not cause statistically significant changes in *TJP1* expression in either TECs or MVECs. However, comparative analysis showed significant differences in basal levels: *TJP1* expression in MVECs was 1.2-fold lower compared with TECs ($p < 0.01$). After incubation with LDL, this difference persisted, with *TJP1* expression in MVECs remaining 1.9-fold lower than in TECs ($p < 0.01$) (Figure 4k).

No statistically significant differences were observed for *OCN* expression. The expression of this gene remained stable in both TECs and MVECs, regardless of LDL exposure (Figure 4i).

2.4.4. Vascular Tone Regulation and Stress Response

Incubation with LDL did not lead to statistically significant changes in *ENOS* expression in either TECs or MVECs. Transcription levels remained comparable between the control and experimental groups. Comparative analysis also did not reveal significant differences in *ENOS* expression between the two cell types (Figure 4e).

In TECs, incubation with LDL was accompanied by a significant increase in *EDN1* expression, which was 2.3-fold higher than in the control without LDL. A similar trend was observed in MVECs, where incubation with LDL resulted in a 1.5-fold increase in *EDN1* transcription relative to basal levels. Comparative analysis showed that basal *EDN1* expression in TECs was significantly higher than in MVECs, by 6.9-fold. After incubation with LDL, this difference further increased: *EDN1* expression in TECs exceeded that in MVECs by 14.5-fold (Figure 4d).

In TECs, LDL exposure caused a moderate increase in *SOD1* expression, which was 1.2-fold higher compared with the control without LDL. A similar trend was observed in MVECs: after incubation with LDL, *SOD1* transcription increased 1.1-fold relative to baseline values. Comparative analysis showed that basal *SOD1* expression in TECs was 1.8-fold higher than in MVECs. After LDL exposure, this difference persisted, with *SOD1* expression in TECs remaining 1.9-fold higher than in MVECs (Figure 4j).

2.5. Analysis of Protein Secretion in the Culture Medium by Mass Spectrometry

Under control conditions, the level of vWF secretion in the MVECs culture medium was 1.15-fold lower than in TECs ($p < 0.05$). A similar ratio was observed under LDL exposure: vWF secretion by MVECs remained approximately 1.18-fold lower compared with TECs ($p < 0.05$) (Figure 5a). Thus, the formation of a multinucleated phenotype was associated with reduced vWF secretion regardless of lipid load.

Under control conditions, VEGFC (vascular endothelial growth factor C) secretion by MVECs was reduced by approximately 1.08-fold compared with TECs ($p < 0.05$). Upon incubation with LDL, the differences between the cell types persisted: VEGFC levels in the MVECs culture medium remained about 1.08-fold lower than in TECs ($p < 0.05$) (Figure 5b).

Under control conditions, *EDN1* secretion by MVECs was 1.32-fold lower than in TECs ($p < 0.05$). After LDL exposure, the differences between the cell types were maintained: *EDN1* levels in the MVECs culture medium remained 1.26-fold lower compared with TECs ($p < 0.05$) (Figure 5c). Thus, MVECs were characterized by consistently lower endothelin-1 secretion irrespective of culture conditions.

In contrast to the other analyzed proteins, t-PA (tissue-type plasminogen activator) secretion under control conditions was increased in MVECs by 1.06-fold compared with TECs ($p < 0.05$). A similar pattern was observed under LDL exposure: t-PA levels in the MVECs culture medium exceeded those in TECs by 1.07-fold ($p < 0.05$) (Figure 5d). This indicates enhanced t-PA secretion by multinucleated endothelial cells regardless of lipid load.

Descriptive statistics of secreted proteins identified by DIA-LC-MS and effect size analysis are presented in Tables S1 and S2 (Supplementary Materials).

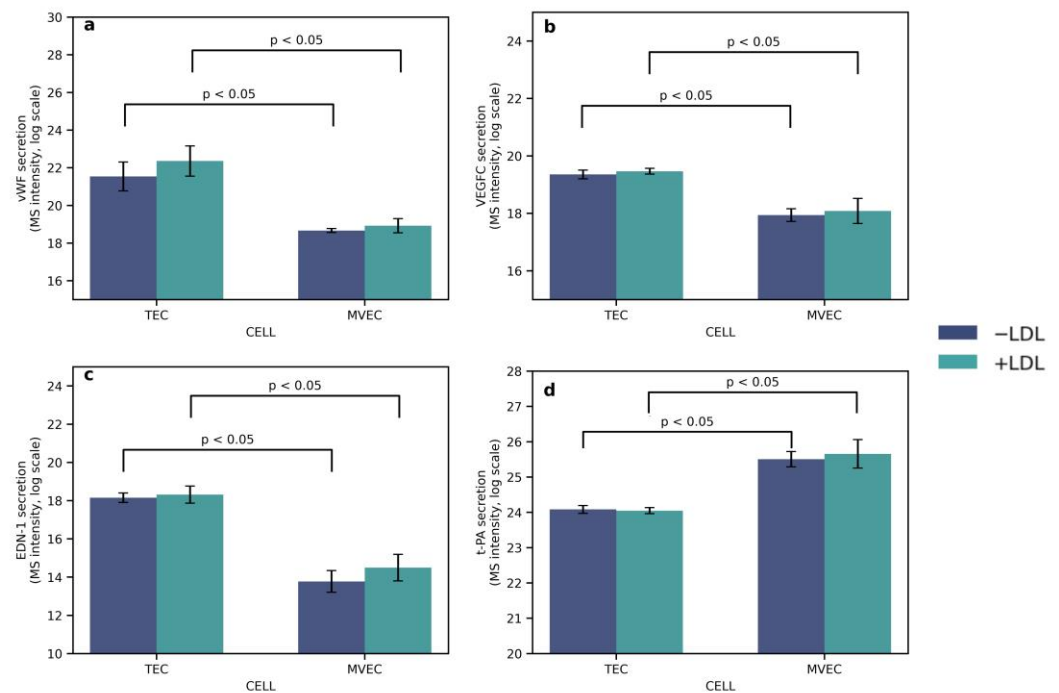


Figure 5. Secretion of endothelial functional markers in conditioned media of TECs and MVECs. (a) von Willebrand factor (vWF), (b) vascular endothelial growth factor C (VEGFC), (c) endothelin-1 (EDN1), (d) tissue-type plasminogen activator (t-PA). Cells were cultured under control conditions (−) or in the presence of LDL (+). Data are presented as mean $\pm$ SEM (n = 3). Statistical significance between TECs and MVECs within the same condition was assessed using one-way ANOVA followed by Tukey's post hoc test; $p < 0.05$.

2.6. Immunocytochemical Analysis of Intercellular Junctions

Immunocytochemical analysis of intercellular junctions was performed for TECs and MVECs under control conditions and after exposure to low-density lipoproteins. In all experimental groups, a confluent monolayer of endothelial cells was formed without signs of compromised integrity. Phase-contrast microscopy was used to confirm the preservation of the monolayer as well as to verify the multinucleated phenotype of MVECs (Figure 6).

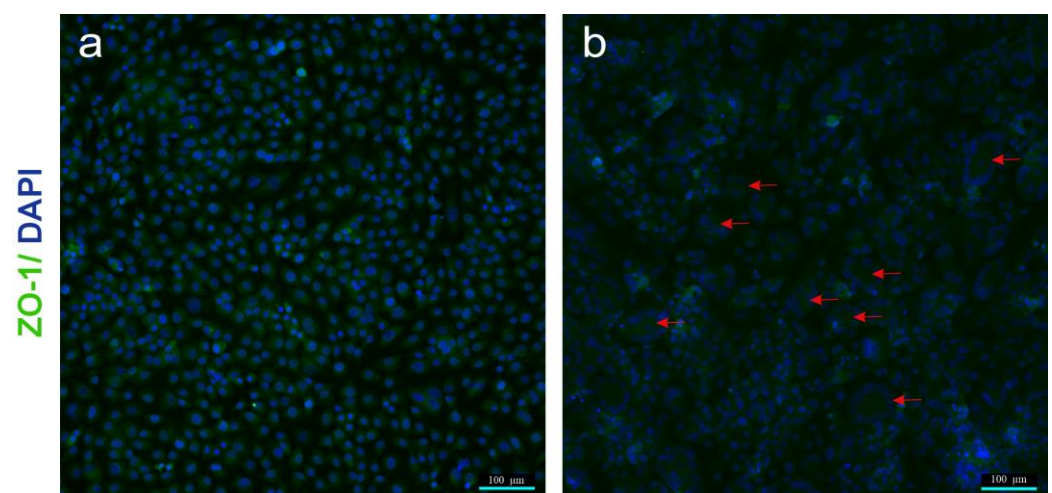


Figure 6. Distribution of the tight junction protein ZO-1 in typical (a) and multinucleated endothelial cells (b). ZO-1 (green), DAPI (blue). Phase-contrast microscopy was used to confirm the multinucleated phenotype of MVECs (indicated by red arrows) and the integrity of the confluent monolayer. Scale bar, 100 μ m.

In TECs, staining of the tight junction protein ZO-1 (zonula occludens-1) was observed predominantly at intercellular contacts, resulting in a pronounced and continuous labeling along the borders between adjacent cells. However, ZO-1 was also detected in the cytoplasm, although the staining intensity was lower than that observed at intercellular junctions.

In MVECs, the distribution of ZO-1 differed from that in TECs. The ZO-1 signal was predominantly localized in the cytoplasm, with a more pronounced concentration in the central region of the cell near the nuclei. At the cell periphery and at intercellular junctions, the intensity of the ZO-1 signal was markedly reduced or almost absent. These distribution patterns were observed both under control conditions and after exposure to low-density lipoproteins.

To provide quantitative confirmation of these observations, fluorescence intensity analysis was performed using Fiji/ImageJ (version 1.54p; National Institutes of Health, Bethesda, MD, USA) (<https://imagej.org>, accessed on 18 February 2026). For each group, 12 identical regions of interest (ROIs) of equal area (254,016 pixels) were analyzed under identical acquisition settings. Mean fluorescence intensity per field was extracted and used as an independent measurement. Quantitative analysis demonstrated that mean ZO-1 fluorescence intensity was significantly lower in MVECs compared with TECs (2.99 ± 0.75 vs. 7.87 ± 0.67 , $p < 0.001$).

3. Discussion

In the course of a pilot study of aortic endothelium samples from patients with aneurysms, we identified areas containing MVECs. This phenomenon has previously been described by other researchers: MVECs were observed in areas of thickened intima and atherosclerotic plaques [4,7], as well as in the microvessels of grafts [6]. Our results confirm that MVECs can form in the vascular wall under pathological conditions; however, due to the limited number of samples, we cannot yet confirm the association of these cells with thoracic aortic aneurysm.

The temporal relationship between the formation of MVECs and vascular injury remains unclear. It cannot be determined whether multinucleation represents a primary event or develops secondary to chronic endothelial damage. Earlier studies have proposed that MVECs arise predominantly through cell fusion rather than incomplete cell division. Nevertheless, the mechanisms underlying their formation in pathological vascular regions remain incompletely defined.

Despite the large number of studies using endothelial models, it remains unclear how cellular heterogeneity affects pathological processes. MVECs have been described in the vascular wall in atherosclerosis and chronic inflammation, but their functional role is still uncertain. In vivo studies of MVECs are difficult: these cells occur focally, their number is small, and biopsy material is limited. Moreover, multinucleated cells do not divide, which makes it impossible to passage them. Therefore, to analyze the functional features of MVECs, we developed an experimental laboratory model based on the EA.hy926 cell line, which was previously used in our work to study transendothelial lipoprotein transport and cell migration [8]. This approach allowed us to reproduce the multinucleated phenotype under controlled conditions and compare its characteristics with typical endothelial cells.

It is important to note that the EA.hy926 line used in this study has a number of features that must be considered when interpreting the results. This line is a hybrid of HUVEC and A549 lung carcinoma cells, which ensures preservation of the basic properties of ECs, but also introduces some differences in the expression profile, aging markers, and other characteristics [9,10]. Thus, the EA.hy926-based model makes it possible to identify differences between TECs and MVECs, but does not fully reflect the heterogeneity and

complexity of the endothelium in vivo. However, we showed that in our model the expression of endothelial markers (CD31, CD146, vWF) was preserved and did not change during cell fusion, confirming maintenance of the basic endothelial phenotype. Additionally, mass spectrometry-based secretome analysis demonstrated that vWF was actively secreted by both TECs and MVECs, and its detection in the extracellular medium confirms the functional integrity of the endothelial secretory apparatus in the experimental model used. This can be considered an advantage of the developed system, as it allows the study of functional differences in multinucleated cells without loss of their endothelial nature.

In vivo, MVECs are rare, occur focally, and cannot be expanded in culture. Therefore, PEG-induced fusion was used to reproducibly generate multinucleated cells and to evaluate how increased nuclear content affects lipid accumulation, inflammatory response, intercellular junction organization, and protein secretion. At the same time, this model does not recapitulate the multicellular and biomechanical complexity of the vascular wall in vivo, and further studies using primary endothelial cells and human tissue samples are required.

In our study, we demonstrated that both TECs and MVECs accumulate cholesterol, but MVECs did so more actively. Lipid overload of the endothelium is one of the early triggers of inflammatory activation and the formation of atherosclerotic plaques [11]. The increased ability of MVECs to accumulate cholesterol may be critical for their phenotype, as excess lipid content in ECs is directly linked to the activation of proinflammatory signaling pathways, such as LOX-1/NF- κ B [11,12].

Increased expression of *NFKB1* in MVECs can be considered a key link connecting lipid overload with inflammatory activation. This transcription factor regulates the expression of genes encoding a wide range of inflammatory mediators, including IL-6, IL-8, and TNF- α , as well as adhesion molecules, which promote the recruitment and migration of monocytes and lymphocytes [13,14]. Thus, at the level of signaling cascades, MVECs display features of a preactivated state characterized by readiness for increased production of proinflammatory factors. Our results confirm this: incubation with LDL caused a marked increase in IL-6 and TNF- α secretion, indicating endothelial proinflammatory activation. At the same time, IL-8 dynamics had distinctive features: in both cell types its level decreased under LDL stimulation, but in MVECs IL-8 secretion remained higher than in TECs both at baseline and after stimulation. Collectively, high basal production of IL-6 and TNF- α , combined with persistently elevated IL-8 levels, defines a pronounced proinflammatory phenotype in MVECs.

Another proinflammatory cytokine of interest in the context of our study is IL-1 β . According to ELISA, IL-1 β secretion did not differ between TECs and MVECs and was not altered by LDL exposure. However, qPCR analysis showed higher IL-1 β expression in MVECs than in TECs, and in both cell types expression increased upon LDL stimulation. This discrepancy between ELISA and qPCR results may reflect post-transcriptional and post-translational regulation of IL-1 β . It is well established that IL-1 β synthesis, processing, and secretion require additional activation signals, including inflammasome-mediated cleavage by caspase-1 [15]. Therefore, increased *IL1B* mRNA levels do not necessarily result in elevated extracellular cytokine levels. In the present study, inflammasome activation was not directly assessed.

It is well established that activation of NF- κ B and proinflammatory cytokines is associated with disruption of endothelial barrier integrity through suppression of tight junction protein expression [16,17]. The elevated levels of proinflammatory mediators and NF- κ B activity observed in MVECs in our study are likely to contribute to decreased expression of tight junction proteins and the formation of local areas of endothelial dysfunction. Indeed, basal *TJP1* expression in MVECs was lower than in TECs, and this relationship persisted

after LDL exposure. This indicates a weakening of intercellular junctions in MVECs, which may have several consequences for the endothelium and vasculature as a whole. When intercellular contacts are weakened, endothelial permeability increases and the migration of immune cells is facilitated, thereby enhancing inflammatory processes in the vascular wall. Disruption of the barrier function also promotes the penetration of leukocytes, monocytes, and circulating lipids into the intima, accelerating the formation of atherosclerotic plaques. Thus, the decreased *TJP1* expression detected in MVECs may reflect their predisposition to junctional instability and can be considered an additional mechanism contributing to endothelial dysfunction.

Additional confirmation of the observed transcriptional changes was obtained by analyzing the subcellular distribution of the tight junction protein ZO-1 using immunocytochemical staining. ZO-1 (zonula occludens-1) is a key component of tight junctions in endothelial cells; it is encoded by the *TJP1* gene and is predominantly localized at the cell membrane, although it is also present in the cytoplasm [18,19].

In TECs, ZO-1 was mainly localized at intercellular junctions, forming a continuous staining pattern along the borders between adjacent cells, while a cytoplasmic signal was also detected. In contrast, in multinucleated endothelial cells, ZO-1 exhibited predominantly cytoplasmic localization with accumulation in the central regions of the cell, in close proximity to the nuclei, whereas the ZO-1 signal at the cell periphery and at intercellular junctions was markedly reduced or nearly absent.

The results obtained at the transcriptional and protein levels were consistent with and complementary to each other. According to PCR data, *TJP1* (encoding ZO-1) expression in MVECs was reduced compared with TECs under both control conditions and after LDL incubation. Immunocytochemical analysis confirmed that the differences between the cell types involved not only expression levels but also the intracellular localization of ZO-1. These findings are consistent with literature reports describing alterations in tight junction proteins during inflammatory activation of the endothelium [17,20].

In our study, we demonstrated that the expression of the secreted endothelial marker von Willebrand factor (vWF) was significantly lower in MVECs compared with EA.hy926 cells under both control conditions and after LDL exposure. vWF is not only a key regulator of hemostasis but also actively participates in the modulation of vascular inflammation [21,22]. Experimental and clinical data indicate that elevated vWF levels are a marker of endothelial dysfunction and increased cardiovascular risk [23,24]. However, endothelial cells are known to exhibit pronounced phenotypic heterogeneity, including variability in vWF expression [25]. Thus, reduced vWF expression in MVECs may reflect a phenotypic shift characterized by a decreased capacity for thrombogenesis and barrier function, accompanied by an enhanced pro-inflammatory potential. Mass spectrometry-based secretome analysis was consistent with the PCR data and showed that vWF secretion by MVECs was also reduced compared with TECs under both control conditions and LDL exposure. Despite the concordant direction of changes, the decrease in vWF expression in MVECs was more pronounced than the reduction in its secretion. This may be related to specific features of vWF synthesis and release regulation in endothelial cells. vWF is synthesized and stored in specialized endothelial secretory granules and can be released into the extracellular space independently of the current transcriptional level.

Regulation of vascular tone is largely determined by the balance between nitric oxide (NO) production and the expression of vasoactive mediators. In our study, eNOS expression remained stable regardless of cell type or LDL exposure. However, eNOS activity and NO production may be altered at the post-translational level. This is supported by numerous studies demonstrating post-translational regulation of nitric oxide synthase, including protein-protein interactions, phosphorylation, S-nitrosylation, acetylation, and changes in

subcellular localization [26]. Therefore, our results do not exclude the possibility of altered NO production mediated by post-translational modifications of eNOS, particularly under conditions of lipid load.

Along with NO, endothelin-1 (EDN1) is a critical regulator of vascular tone and one of the most potent vasoconstrictors produced by the endothelium. It plays a key role in vascular remodeling and the progression of atherosclerosis, and its expression is enhanced by LDL and pro-inflammatory cytokines [27,28]. In our study, EDN1 expression was significantly higher in TECs compared with MVECs, with LDL exposure leading to a more pronounced increase specifically in TECs. Mass spectrometry-based secretome analysis was consistent with the PCR data and demonstrated that EDN1 secretion by MVECs was also reduced compared with TECs under both control conditions and LDL exposure. The concordant direction of changes in expression and secretion indicates an overall reduction in the functional contribution of endothelin-1 to the secretory profile of multinucleated endothelial cells. These results suggest that TECs are more strongly involved in the regulation of vascular tone, whereas MVECs exhibit a shift in functional activity toward inflammatory processes. Given that LDL exposure increased EDN1 expression in both cell types, it can be assumed that lipid load stimulates endothelin-1 hyperexpression, although this is not accompanied by increased secretion, which may contribute to endothelial dysfunction and vascular remodeling [27,29]. However, since the present study evaluated transcript and secreted protein levels only, without direct assessment of receptor signaling or nitric oxide production, conclusions regarding functional differences in vascular tone should be interpreted with caution.

Regulation of vascular tone represents only one of the many endothelial functions; processes of vascular remodeling and endothelial adaptation to injury are equally important. In this context, alongside endothelin-1, factors involved in angiogenesis and extracellular matrix remodeling are of particular interest.

VEGFC is one of the key regulators of vascular remodeling and endothelial plasticity, contributing to the maintenance of vascular wall structure and regulation of endothelial permeability [30]. In our study, mass spectrometry-based secretome analysis revealed reduced VEGFC secretion by MVECs compared with TECs under both control conditions and LDL exposure. This may indicate an impairment of the angiogenic function of MVECs and is consistent with previously identified alterations in endothelial barrier properties.

At the same time, MVECs exhibited changes in the secretion of proteins associated with extracellular matrix remodeling. In particular, mass spectrometry data showed that secretion of tissue-type plasminogen activator (t-PA) was increased in MVECs compared with TECs, regardless of culture conditions. t-PA plays a central role in the regulation of fibrinolysis and proteolytic processes that mediate extracellular matrix degradation and cell migration [31]. Excessive activation of the t-PA/plasmin system is known to promote extracellular matrix degradation, compromise vascular wall structural stability, and enhance inflammatory processes [32]. Thus, increased t-PA secretion by MVECs may represent one of the mechanisms contributing to endothelial dysfunction and vascular structural instability.

Taken together, the combination of reduced VEGFC secretion and increased t-PA production in MVECs indicates an imbalance between vascular adaptive processes and extracellular matrix degradation, which may contribute to the progression of endothelial dysfunction.

Special attention should also be paid to antioxidant defense, which plays a key role in maintaining vascular homeostasis and preventing the accumulation of reactive oxygen species [33]. One of the main enzymes of this system is superoxide dismutase-1 (SOD1). Reduced SOD1 activity or expression is known to enhance oxidative stress and promote

endothelial dysfunction [33]. In our study, SOD1 expression was lower in MVECs compared with TECs, both under basal conditions and after LDL incubation. These data indicate an intrinsically lower antioxidant potential of multinucleated cells. Reduced antioxidant defense, together with increased pro-inflammatory cytokine expression, may contribute to endothelial dysfunction and vascular wall damage.

Thus, the endothelium represents a heterogeneous tissue in which, alongside typical endothelial cells, atypical phenotypes such as MVECs can emerge. Their presence has been described in atherosclerosis, inflammation, and other pathological conditions; however, their functional role remains poorly understood. In this work, a laboratory model based on EA.hy926 cells enabled reproduction of the multinucleated phenotype and its comparison with typical endothelial cells. The results showed that MVECs are characterized by enhanced cholesterol accumulation, increased expression of NF- κ B and pro-inflammatory cytokines, and reduced antioxidant potential. These findings suggest that MVECs may act as a source of chronic inflammation within the vascular wall, and their investigation represents an important step toward understanding the mechanisms of endothelial dysfunction and identifying new therapeutic targets. A promising direction for future research will be the transition from the EA.hy926-based model to primary endothelial cells, including HUVECs and cells isolated directly from the aorta. This will allow more precise assessment of the contribution of MVECs to the pathogenesis of vascular diseases and improve the clinical relevance of the obtained data.

Although the EA.hy926-based PEG fusion model does not fully reproduce endothelial heterogeneity in vivo, it enables controlled assessment of how multinucleation influences endothelial function. The inflammatory and junction-related changes observed in MVECs represent novel findings that warrant further validation in primary endothelial cells and in vivo models.

4. Materials and Methods

4.1. Experimental Design

Both TECs (EA.hy926) and MVECs were used in parallel experiments. After generation of MVECs through PEG-induced fusion, both cell types were seeded at equal densities and cultured to confluence under identical conditions. Experimental groups included cells incubated either in standard culture medium (control) or in medium supplemented with 100 μ g/mL of isolated LDL. Incubation was carried out for 24 h at 37 °C in a humidified atmosphere with 5% CO₂.

Following incubation, cells and culture supernatants were collected for analysis. The experimental workflow comprised three major parts:

1. Biochemical analysis—determination of intracellular cholesterol accumulation using the Folch method with normalization to total protein.
2. Cytokine secretion—quantification of IL-1 β , IL-6, IL-8 and TNF- α levels in culture supernatants by ELISA.
3. Gene expression profiling—assessment of transcriptional changes in selected inflammatory, endothelial, and barrier-related markers (*IL-1 β* , *IL6*, *IL8*, *NFKB1*, *CD31*, *CD146*, *VWF*, *OCLN*, *TJP1*, *eNOS*, *EDN1*, *SOD1*, *ACTB*) by quantitative real-time PCR (qPCR).
4. Immunocytochemical analysis—evaluation of intercellular junction organization by immunocytochemical staining of the tight junction protein ZO-1 in confluent monolayers of TECs and MVECs under control conditions. Phase-contrast microscopy was additionally used to confirm monolayer integrity and the multinucleated phenotype of MVECs.
5. Secretome profiling—analysis of proteins secreted into the culture medium by TECs and MVECs using data-independent acquisition liquid chromatography–tandem

mass spectrometry (DIA-LC-MS). Culture supernatants were collected, processed, and subjected to mass-spectrometric analysis to characterize changes in the extracellular protein profile associated with the multinucleated endothelial phenotype and LDL exposure.

4.2. Cell Culture

Human endothelial EA.hy926 cells (ATCC, Manassas, VA, USA) were used as a model of TECs. This line exhibits a confirmed endothelial phenotype, including the expression of the characteristic marker vWF. Despite their wide application in vascular biology research, EA.hy926 represents a hybrid (HUVEC $\times$ A549 carcinoma), which should be considered as a limitation of the model. Cells were maintained in DMEM/F12 medium (PanEco, Moscow, Russia) supplemented with 10% fetal bovine serum (FBS, Biosera, Nuaille, France) at 37 °C in a humidified atmosphere with 5% CO₂.

4.3. Induction of Multinucleated Endothelial Cells

To obtain MVECs, confluent monolayers of EA.hy926 were subjected to three consecutive treatments with a 50% polyethylene glycol (PEG) 6000 solution (PanEco, Moscow, Russia), which induced membrane fusion. This mechanism has been well described in classical cell hybridization protocols [34]. With short incubation and careful washing, cells were allowed to recover for not less than 24 h after the final PEG treatment before functional assays were initiated. Previous studies have shown that PEG-mediated fusion may induce transient stress responses and membrane remodeling [35–37]. In the present study, all downstream analyses were conducted after morphological recovery of the monolayer. Cells were cultured in DMEM/F12 medium supplemented with 10% FBS at 37 °C in a humidified atmosphere with 5% CO₂.

Multinucleated endothelial cells covered not less than 50% of the total culture surface area 24 h after PEG-induced fusion. Nuclear number was quantified in four independent microscopic fields. A total of 61 multinucleated cells were analyzed, with a mean of 8.2 ± 3.6 nuclei per cell (range 3–16).

4.4. Endothelial Prints from Thoracic Aortic Tissue

Postoperative thoracic aortic wall tissue was used to prepare endothelial prints. The study included patients (n = 4) diagnosed with thoracic aortic aneurysm who underwent surgical treatment, which included work with aortic tissues and which included removal of pathologically altered aortic segments. The tissue was placed in a phosphate-buffered saline (PBS) solution (PanEco, Moscow, Russia) and several buffer changes were performed to remove blood. The adventitia was removed and rinsed in PBS. The fragment was placed on filter paper with the luminal side facing up. A gauze cloth folded in four was applied and gently pressed to remove the liquid. The preparations were left to dry at room temperature. When the intima became opaque, the preparation of the EC monolayer was prepared. The quality of the print was visually monitored with a light microscope and left to air dry for 24 h. Then the samples were fixed in 4% paraformaldehyde (PFA) (PanEco, Moscow, Russia) and stained with DAPI (BioFroxx, Einhausen, Germany).

A total of four endothelial prints were obtained from thoracic aortic aneurysm specimens derived from four different patients (one print per patient). Two prints were collected from aneurysmal segments and two from macroscopically intact aortic regions distant from the lesion.

MVECs were considered present when cells with clearly identifiable 3 or more distinct DAPI-positive nuclei within a single continuous cytoplasmic contour were observed in the endothelial monolayer. Only cells exceeding the size of typical endothelial cells

(approximately ≥ 3 -fold larger surface area) and containing ≥ 3 nuclei were classified as MVECs.

4.5. LDL Isolation

LDL was isolated from human plasma by sequential density-gradient ultracentrifugation, as previously described [11]. Density solutions were prepared as follows: 1.006 g/cm³ NaCl/EDTA (11.42 g/L NaCl, 0.1 g/L EDTA in Milli-Q water) (PanEco, Moscow, Russia); 1.019 g/cm³ (16.5 g/L KBr (DIA-M, Moscow, Russia) in 1.006 g/cm³ NaCl/EDTA); 1.065 g/cm³ (77.1 g/L KBr in 1.006 g/cm³ NaCl/EDTA).

Crystalline KBr (0.5 g per 1 mL of plasma) was added to collected plasma and dissolved with gentle vortexing. The saline plasma was transferred into ultracentrifuge tubes to two-thirds of their final volume, and the remaining one-third was filled with the 1.019 g/cm³ KBr solution. Tubes were balanced (tolerance ± 0.01 g) and centrifuged at 40,000 rpm for 50 min at +4 °C using a pre-cooled rotor (Beckman L8-55M, Brea, CA, USA). After centrifugation, chylomicrons and HDL accumulated in the upper layer, whereas LDL remained at the phase separation boundary. The upper layer was removed, leaving 1–2 mm above the boundary. A 1.065 g/cm³ KBr solution was carefully layered on top (volume adjusted according to the amount of removed supernatant, ~4 mL for an initial 21 mL load). The tubes were rebalanced and centrifuged for 2 h 10 min at 40,000 rpm and +4 °C. After ultracentrifugation, LDL migrated into the upper fraction, forming a reddish-brown ring. The LDL fraction was collected by carefully pipetting at the ring level. The study was approved by the Local Ethics Committee of the National Research Center for Surgery Petrovsky (Approval No. 3 dated 17 March 2022).

4.6. Cell Seeding and LDL Treatment

TECs and MVECs were seeded into 24-well plates at a density of 5×10^5 cells per 1 mL of DMEM/F12 medium supplemented with 10% FBS. After reaching 100% confluence, the culture medium was replaced with DMEM/F12 containing 100 µg/mL of purified LDL. Cells were incubated for 24 h at 37 °C in 5% CO₂. Culture supernatants were collected and stored at −80 °C for subsequent ELISA analysis.

The selected concentration (100 µg/mL) and incubation period (24 h) correspond to commonly used experimental conditions for modeling LDL-induced endothelial dysfunction in vitro and were chosen to ensure sufficient lipid accumulation while avoiding overt cytotoxicity [11,12]. LDL preparations were native (non-oxidized) human LDL obtained by sequential density-gradient ultracentrifugation.

4.7. RNA Isolation

Total RNA was isolated using the RUpplus RNA extraction kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer's protocol. After removal of the culture medium, lysis buffer was added directly to the cells in the wells, followed by incubation for 10 min at room temperature. The lysate was collected and transferred to microtubes.

Lysates were centrifuged for 10 min at 10,000× *g*, and the supernatant was transferred to clean 1.5–2 mL tubes. To each sample, 400 µL of binding buffer was added, mixed by pipetting, and up to 800 µL of the mixture was applied onto the spin column. Columns were centrifuged for 30 s at 10,000× *g*, and the flow-through was discarded. If the total sample volume exceeded 800 µL, the remaining fraction was reapplied to the same column and centrifuged under the same conditions.

Columns were washed sequentially with 500 µL of wash buffer 1 and 500 µL of wash buffer 2 (supplemented with ethanol), each followed by centrifugation for 30 s at 10,000× *g*. Finally, columns were centrifuged for 3 min at 10,000× *g* to remove residual WB2.

Columns were placed into clean 1.5–2 mL tubes, and 60–200 µL of elution buffer was applied directly to the center of the membrane. After 1 min incubation at room temperature, RNA was eluted by centrifugation for 1 min at $10,000\times g$.

The concentration and purity of isolated RNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was verified by agarose gel electrophoresis. Samples were stored at $-80\text{ }^{\circ}\text{C}$ until use for cDNA synthesis.

4.8. cDNA Synthesis

First-strand cDNA was synthesized using the M-MuLV–RH reverse transcriptase kit (Biolabmix, Novosibirsk, Russia) according to the manufacturer’s instructions. Briefly, 0.1 ng–5 µg of total RNA was mixed with 1–3 µL of primers in nuclease-free water to a final volume of 12 µL. The mixture was heated at $70\text{ }^{\circ}\text{C}$ for 2–3 min to denature secondary RNA structures and immediately chilled on ice.

A reaction mix containing 4 µL of $5\times$ RT buffer, 1 µL of M-MuLV–RH reverse transcriptase (100 U/µL), and 3 µL of nuclease-free water was then added (final volume 20 µL). Reactions were incubated at $25\text{ }^{\circ}\text{C}$ for 10 min followed by 60 min at $42\text{ }^{\circ}\text{C}$.

Reverse transcription was terminated by heating to $70\text{ }^{\circ}\text{C}$ for 10 min. The resulting cDNA was either used immediately for qPCR or stored at $-20\text{ }^{\circ}\text{C}$ for short-term use and at $-70\text{ }^{\circ}\text{C}$ for long-term storage.

4.9. Quantitative Real-Time PCR (qPCR)

Quantitative PCR was performed using the commercial reaction mixture $5\times$ qPCRmix-HS SYBR (Evrogen, Moscow, Russia) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instructions. Each reaction was carried out in a final volume of 25 µL, containing 2 µL of cDNA template, 5 µL of $5\times$ qPCRmix-HS SYBR, 0.4 µM of each primer, and nuclease-free water.

Amplification conditions were as follows: initial denaturation at $95\text{ }^{\circ}\text{C}$ for 3 min, followed by 40 cycles of $95\text{ }^{\circ}\text{C}$ for 10 s and $60\text{ }^{\circ}\text{C}$ for 30 s. Melt curve analysis was performed after amplification to verify the specificity of the PCR products.

For real-time PCR, primers specific to the following genes were used: *ACTB* (β -actin), *TJP1* (tight junction protein 1), *SOD1* (superoxide dismutase-1), *VWF* (von Willebrand factor), *ENOS* (endothelial nitric oxide synthase), *EDN1* (preproendothelin-1), *IL1B* (interleukin 1 beta), *NFKB1* (nuclear factor kappa-light-chain-enhancer of activated B cells), *CD31* (cell adhesion marker), *CD146* (cell adhesion marker), *OCN* (occludin), *IL8* (interleukin 8) and *IL6* (interleukin 6).

The sequences of forward and reverse primers are provided in Table 1. Primers were synthesized by Lumiprobe (Moscow, Russia), and their specificity was validated using the Primer-BLAST (NCBI) service (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>, accessed on 14 April 2025). Relative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method using *ACTB* as the internal control.

Table 1. Primer sequences for real-time PCR expression evaluation.

The Gene	Forward Primer	Reverse Primer
<i>ACTB</i>	CACCATTTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
<i>TJP1</i>	GTCCAGAATCTCGGAAAAGTGCC	CTTTCAGCGCACCATACCAACC
<i>SOD1</i>	CTCACTCTCAGGAGACCATTGC	CCACAAGCCAAACGACTTCCAG
<i>VWF</i>	CCTTGAATCCCAGTGACCCTGA	GGTCCGAGATGTCTCCACAT
<i>ENOS</i>	GAAGGCGACAATCCTGTATGGC	TGTTTCGAGGGACACCACGTCAT
<i>EDN1</i>	CTACTTCTGCCACCTGGACATC	TCACGGTCTGTTGCCTTTGTGG

Table 1. Cont.

The Gene	Forward Primer	Reverse Primer
<i>IL1B</i>	AGCTCGCCAGTGAAATGATG	GGTGGTCGGAGATTCGTAGC
<i>NFKB1</i>	TGGGAAGGCCTGAACAAATG	TATGGGCCATCTGTTGGCAG
<i>CD31</i>	AAGTGGAGTCCAGCCGCATATC	ATGGAGCAGGACAGGTTTCAGTC
<i>CD146</i>	ATCGCTGCTGAGTGAACCACAG	CTACTCTCTGCCCTCACAGGTCA
<i>OCLN</i>	CACACAGGACGTGCCTTCAC	GGCTGCCTGAAGTCATCCAC
<i>IL8</i>	GAGAGTGATTGAGAGTGGACCAC	CACAACCCTCTGCACCCAGTTT
<i>IL6</i>	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG

4.10. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of secreted cytokines TNF- α , IL-1 β , IL-6 and IL-8 were measured using the following commercial enzyme immunoassay kits (ELISA): Human TNF- α /TNFSF1ADuoSet ELISA, Human IL-1 beta/IL-1F2 DuoSet ELISA, Human IL-6 DuoSet ELISA, Human IL-8/CXCL8 DuoSet ELISA (R&D Systems Inc., Minneapolis, MN, USA).

The analysis was performed in 96-well plates with high protein binding capacity. The plates were pre-coated with a primary antibody diluted in PBS to the manufacturer's recommended concentration, and incubated at room temperature overnight. After incubation, the wells were washed three times with PBS with 0.05% Tween-20 (PanEco, Moscow, Russia) (PBS-T) and blocked with 1% BSA (bovine serum albumin) (PanEco, Moscow, Russia) diluted in PBS at room temperature for 2 h.

100 μ L of test samples or standards, diluted in 1% BSA/PBS, was added to each well. Incubation was carried out at room temperature for 2 h (all subsequent incubations were carried out on a shaker at 200 rpm in the dark). After repeated washing, a secondary antibody was added, diluted according to the instructions, and incubated for 2 h at room temperature. Then PBS-T was washed and streptavidin conjugated with horseradish peroxidase (HRP) was added and incubated for 20 min.

TMB substrate (3,3',5,5'-tetramethylbenzidine) was used for detection. The reaction was stopped by adding 2 M H₂SO₄. The optical density was measured at a wavelength of 450 nm with a correction of 540 nm using a microplate photometer. Cytokine concentrations were calculated using calibration curves based on the standards included in the kits.

4.11. Immunocytochemical Analysis of Intercellular Junctions

Immunocytochemical analysis of intercellular junctions was performed for TECs (EA.hy926) and MVECs under control conditions.

Cells were seeded onto coverslips and cultured until a confluent monolayer was formed. After completion of the culture period, cells were fixed in 4% PFA in PBS for 15 min at room temperature and subsequently washed with PBS.

Nonspecific binding was blocked by incubating the samples in a solution containing 1% BSA in PBS for 30 min at room temperature. The samples were then incubated with a primary antibody anti-ZO-1 tight junction protein (Servicebio, Wuhan, China), diluted in the blocking solution, overnight at 4 °C. After incubation, the specimens were washed with PBS and incubated with a fluorescently labeled secondary antibody (Abcam, Cambridge, UK) for 1 h at room temperature in the dark.

Cell nuclei were counterstained with DAPI. After staining, the samples were washed with PBS and mounted using an antifade mounting medium.

Imaging was performed using a fluorescence microscope under identical exposure settings for all samples. Phase-contrast microscopy was used to confirm monolayer integrity and to identify the multinucleated phenotype of MVECs. The distribution of ZO-1 was

analyzed qualitatively, with particular attention to its localization at intercellular junctions and within the cytoplasm of the cells.

Quantitative analysis of ZO-1 fluorescence was performed using Fiji/ImageJ software. For each group, 12 identical regions of interest (ROIs) with equal area were selected per image. Mean fluorescence intensity values were extracted for each ROI. The average intensity per field was used as a unit of analysis for statistical comparison between TECs and MVECs groups.

4.12. Sample Preparation

Protein reduction, alkylation, and enzymatic digestion were performed according to a previously described protocol [38] with minor modifications. Briefly, 10 µg of protein sample was mixed with 10 µL of sodium deoxycholate (SDC, Sigma-Aldrich, St. Louis, MO, USA)-based buffer (pH 8.5) containing 100 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO, USA), 1% (*w/v*) SDC, 10 mM TCEP (Sigma-Aldrich, St. Louis, MO, USA), and 20 mM chloroacetamide (Sigma-Aldrich, St. Louis, MO, USA).

The mixture was sonicated in a water bath sonicator for 1 min, incubated at 85 °C for 10 min, and then cooled to room temperature. Trypsin (Promega, Madison, WI, USA) dissolved in 100 mM Tris-HCl (pH 8.5) was added at an enzyme-to-protein ratio of 1:50 (*w/w*), and samples were incubated overnight at 37 °C for protein digestion.

After digestion, peptides were acidified by adding 50 µL of 2% trifluoroacetic acid (TFA, Sigma-Aldrich, St. Louis, MO, USA) mixed with 50 µL of ethyl acetate (Sigma-Aldrich, St. Louis, MO, USA) and subsequently loaded onto SDB-RPS StageTips containing two 14-gauge disks (Empore, 3M, St. Paul, MN, USA). StageTips were centrifuged at 300× *g* until the solution completely passed through the sorbent (typically ~4 min).

The StageTips were then washed twice with 100 µL of 1% TFA/ethyl acetate (1:1) and once with 50 µL of 0.2% TFA. Peptides were eluted into clean tubes using 60 µL of 60% acetonitrile/5% ammonia solution (Merck, Darmstadt, Germany) by centrifugation at 300× *g*. The eluates were dried under vacuum and stored at −80 °C until analysis.

Prior to LC–MS analysis, peptides were reconstituted in 20 µL of 2% acetonitrile/0.1% TFA and briefly sonicated for 1 min.

4.13. DIA-LC-MS Analysis

Data-independent acquisition liquid chromatography–mass spectrometry (DIA-LC-MS) analysis was performed as described previously [39] with minor modifications. Peptides were loaded onto a home-made trap column (50 × 0.1 mm) packed with Reprosil-Pur 200 C18-AQ resin (5 µm; Dr. Maisch GmbH, Ammerbuch, Germany) using a loading buffer containing 2% acetonitrile (ACN), 98% H₂O, and 0.1% trifluoroacetic acid (TFA) at a flow rate of 4 µL/min.

Peptide separation was carried out at room temperature on a home-packed fused-silica analytical column (300 × 0.1 mm) filled with Reprosil-Pur C18-AQ resin (1.9 µm; Dr. Maisch). The emitter was prepared using a P2000 laser puller (Sutter Instrument, Novato, CA, USA).

Reversed-phase liquid chromatography was performed using an Ultimate 3000 Nano LC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Orbitrap Tribrid Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) via a nano-electrospray ionization source (Thermo Fisher Scientific, Waltham, MA, USA). Mobile phase A consisted of water containing 0.1% (*v/v*) formic acid (FA, Thermo Fisher Scientific, Waltham, MA, USA), and mobile phase B consisted of acetonitrile containing 0.1% FA and 20% (*v/v*) water.

Peptides were eluted from the trap column at a flow rate of 500 nl/min using a linear gradient as follows:

3–6% B for 5 min,
6–35% B for 53 min,
35–60% B for 4 min,
60% B for 6 min,
60–99% B for 0.1 min,
99% B for 7 min,
99–2% B for 0.1 min.

Mass spectrometric data were acquired in DIA mode. For overlapping-window DIA-MS acquisition, MS1 spectra were collected in the m/z range of 495–745 at a resolution of 15,000 with a standard AGC target. MS2 spectra were acquired in the m/z range of 200–1800 at a resolution of 50,000, with a normalized AGC target of 2000%, maximum injection time set to “auto,” and stepped normalized collision energies of 22, 26, and 30%.

The isolation window width was set to 4 Da.

4.14. Statistical Analysis

For PCR and ELISA all experiments were performed using at least six independent biological replicates. For each biological replicate, measurements were carried out in a minimum of two technical replicates. Statistical significance was assessed using the Kruskal–Wallis test and the Conover multiple comparison test. For all results, $p < 0.05$ was considered significant. Statistical data analysis was performed using the Python (version 3.10.4) programming language and the SciPy and scikit-learn libraries.

For mass spectrometry-based secretome analysis we used three independent biological replicates. Before statistical analysis the data were filtered out in order to obtain the list of proteins for the secretome. The methodology was previously published [40] and reproduced by us in previous work [38].

The resulting list of proteins was used for further statistical analysis in Perseus. Only the proteins with valid maxLFQ values in all 2 samples in at least one group were used. Missing values were imputed from normal distribution with 0.3 intensity distribution sigma width and 2.8 intensity distribution center downshift. Statistical significance of differences between experimental groups was assessed using analysis of variance (ANOVA) with permutation-based FDR 5% followed by correction for multiple comparisons using Tukey’s HSD test. Differences were considered statistically significant at $p < 0.05$. Fluorescence intensity values were compared between groups using the Mann–Whitney U test.

Visualization of all results was carried out using standard Python libraries: Seaborn (version 0.13.2), Matplotlib (version 3.8.4).

To select the appropriate statistical test, data normality was assessed using the Shapiro–Wilk test and by visual inspection of quantile–quantile (Q–Q) plots. PCR and ELISA measurements demonstrated deviation from a normal distribution and considerable variability; therefore, the non-parametric Kruskal–Wallis test was applied, as it does not assume normality.

For the mass spectrometry data ($n = 3$ biological replicates), formal testing did not indicate significant deviation from normality. However, with such a small sample size, reliable determination of the underlying distribution is statistically limited. The data exhibited low within-group variability and comparable variances across groups. Considering the relative robustness of parametric methods to moderate deviations from normality and the limitations of non-parametric tests with extremely small sample sizes, we used ANOVA for the analysis of mass spectrometry data.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052425/s1>, All supplementary tables are provided in a single file.

Author Contributions: V.C. and A.M.M., conceptualization and study design; D.B. and E.C., surgical operations and clinical data curation; U.K., V.C. and R.Z., performed the experiments; O.D., resources and collection of clinical plasma samples; D.K., statistical data analysis; T.K., and A.M.M. supervision. All authors discussed data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science and Higher Education of the Russian Federation (FURG-2026-0032).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Local Ethics Committee of the National Research Center for Surgery named after B.V. Petrovsky (protocol code №3, 17 March 2022). Written informed consent was obtained from all subjects involved in the study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ECs	Endothelial cells
TECs	Typical endothelial cells
MVECs	Multinucleated variant endothelial cells
ED	Endothelial dysfunction
LDLs	Low-density lipoproteins
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with tween-20
BSA	Bovine serum albumin
FBS	Fetal bovine serum
PEG	Polyethylene glycol
PFA	Paraformaldehyde
ELISA	Enzyme-linked immunosorbent assay
qPCR	Quantitative real-time polymerase chain reaction
TMB	3,3',5,5'-tetramethylbenzidine
ACTB	β -actin
TJP1	Tight junction protein 1
SOD1	Superoxide dismutase 1
VWF/vWF	von Willebrand factor
ENOS/eNOS	Endothelial nitric oxide synthase
EDN1	Endothelin-1
IL1B/IL-1 β	Interleukin-1 β
IL6/IL-6	Interleukin-6
IL8/IL-8	Interleukin-8
TNF- α	Tumor necrosis factor alpha
NFKB1/NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
CD31 (PECAM1)	Platelet endothelial cell adhesion molecule-1
CD146 (MCAM)	Melanoma cell adhesion molecule
OCLN	Ocludin
t-PA	Tissue-type plasminogen activator

VEGFC	Vascular endothelial growth factor C
EDTA	Ethylenediaminetetraacetic acid
DMEM	Dulbecco's Modified Eagle Medium
DAPI	4',6-Diamidino-2-phenylindole
cDNA	Complementary deoxyribonucleic acid
RNA	Ribonucleic acid
HRP	Horseradish peroxidase
ZO-1	Zonula occludens-1
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
LC-MS	Liquid chromatography–mass spectrometry
FDR	False discovery rate
SEM	Standard error of the mean
HDLs	High-density lipoproteins
ROIs	Regions of interest

References

1. Pepin, M.E.; Gupta, R.M. The Role of Endothelial Cells in Atherosclerosis: Insights from Genetic Association Studies. *Am. J. Pathol.* **2024**, *194*, 499–509. [\[CrossRef\]](#)
2. Dinu, M.; Ruzzolini, J.; Higashi, Y. Roles of Oxidative Stress and Inflammation in Vascular Endothelial Dysfunction-Related Disease. *Antioxidants* **2022**, *11*, 1958. [\[CrossRef\]](#)
3. Feng, Y.; Li, C.; Chen, J.; Xiao, X.; Mao, Q.; Zhao, H.; Wang, J.; Liu, B. Endothelial dysfunction in atherosclerosis: From classical pathways to emerging mechanisms. *Vessel Plus.* **2025**, *9*, 8. [\[CrossRef\]](#)
4. Tokunaga, O.; Fan, J.; Watanabe, T. Atherosclerosis- and age-related multinucleated variant endothelial cells in primary culture from human aorta. *Am. J. Pathol.* **1989**, *135*, 967.
5. Nikiforov, N.; Zlenko, D.; Orekhov, A.N. Giant multinucleated endothelial cells are associated with local accumulation of LDL and CD45+ lymphocytes in the intima of human aorta. *Atherosclerosis* **2022**, *355*, 6. [\[CrossRef\]](#)
6. Zuckerman, J.E.; Brealey, J.; Yabu, J.M.; Chang, A. Large Multinucleated Variant Endothelial Cells in Allograft Kidney Microvasculature: A Biopsy Series. *Kidney Med.* **2022**, *4*, 100411. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Nikiforov, N.G.; Zlenko, D.V.; Orekhova, V.A.; Melnichenko, A.A.; Orekhov, A.N. Local Accumulation of Lymphocytes in the Intima of Human Aorta Is Associated with Giant Multinucleated Endothelial Cells: Possible Explanation for Mosaicism of Atherosclerosis. *Int. J. Mol. Sci.* **2022**, *23*, 1059. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Cherednichenko, V.R.; Khovantseva, U.S.; Kuzmin, V.V.; Chertovich, N.F.; Markin, A.M. Modeling the processes of transendothelial transport of LDL and macrophage migration. *Russ. J. Immunol.* **2024**, *27*, 499–504. [\[CrossRef\]](#)
9. Abdelgawad, I.Y.; Agostinucci, K.; Ismail, S.G.; Grant, M.K.O.; Zordoky, B.N. EA.hy926 Cells and HUVECs Share Similar Senescence Phenotypes but Respond Differently to the Senolytic Drug ABT-263. *Cells* **2022**, *11*, 1992. [\[CrossRef\]](#)
10. Baranska, P.; Jerczynska, H.; Pawlowska, Z.; Koziolkiewicz, W.; Cierniewski, C.S. Expression of Integrins and Adhesive Properties of Human Endothelial Cell Line EA.hy 926—PubMed. *Cancer Genom. Proteom.* **2025**, *2*, 265–269.
11. Kiseleva, D.; Kolmogorov, V.; Cherednichenko, V.; Khovantseva, U.; Bogatyreva, A.; Markina, Y.; Gorelkin, P.; Erofeev, A.; Markin, A. Effect of LDL Extracted from Human Plasma on Membrane Stiffness in Living Endothelial Cells and Macrophages via Scanning Ion Conductance Microscopy. *Cells* **2024**, *13*, 358. [\[CrossRef\]](#)
12. Bogachkov, Y.Y.; Chen, L.; Le Master, E.; Fancher, I.S.; Zhao, Y.; Aguilar, V.; Oh, M.-J.; Wary, K.K.; DiPietro, L.A.; Levitan, I. LDL induces cholesterol loading and inhibits endothelial proliferation and angiogenesis in Matrigels: Correlation with impaired angiogenesis during wound healing. *Am. J. Physiol. Cell Physiol.* **2020**, *318*, C762–C776. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Tak, P.P.; Firestein, G.S. NF- κ B: A key role in inflammatory diseases. *J. Clin. Investig.* **2001**, *107*, 7–11. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Roos, K.; Berkholz, J. LDL Affects the Immunomodulatory Response of Endothelial Cells by Modulation of the Promyelocytic Leukemia Protein (PML) Expression via PKC. *Int. J. Mol. Sci.* **2023**, *24*, 7306. [\[CrossRef\]](#)
15. Dinarello, C.A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunol. Rev.* **2018**, *281*, 8–27. [\[CrossRef\]](#)

16. Yu, H.; Huang, X.; Ma, Y.; Gao, M.; Wang, O.; Gao, T.; Shen, Y.; Liu, X. Interleukin-8 Regulates Endothelial Permeability by Down-regulation of Tight Junction but not Dependent on Integrins Induced Focal Adhesions. *Int. J. Biol. Sci.* **2013**, *9*, 966–979. [\[CrossRef\]](#)
17. Aveleira, C.A.; Lin, C.M.; Abcouwer, S.F.; Ambrósio, A.F.; Antonetti, D.A. TNF- α Signals Through PKC ζ /NF- κ B to Alter the Tight Junction Complex and Increase Retinal Endothelial Cell Permeability. *Diabetes* **2010**, *59*, 2872. [\[CrossRef\]](#)
18. Bauer, H.C.; Traweger, A.; Bauer, H. Proteins of the Tight Junction in the Blood-Brain Barrier. In *Blood-Spinal Cord and Brain Barriers in Health and Disease*; Academic Press: Cambridge, MA, USA, 2004; pp. 1–10. [\[CrossRef\]](#)
19. Haberichter, S.L.; Fahs, S.A.; Montgomery, R.R. Von Willebrand factor storage and multimerization: 2 independent intracellular processes: Presented in abstract form at the 41st Annual Meeting of the American Society of Hematology, New Orleans, LA, USA, 3–7 December 1999. *Blood* **2000**, *96*, 1808–1815. [\[CrossRef\]](#)
20. Capaldo, C.T.; Nusrat, A. Cytokine regulation of tight junctions. *Biochim. Biophys. Acta (BBA)—Biomembr.* **2009**, *1788*, 864–871. [\[CrossRef\]](#)
21. Zifkos, K.; Bochenek, M.L.; Gogiraju, R.; Robert, S.; Pedrosa, D.; Kiouptsi, K.; Moiko, K.; Wagner, M.; Mahfoud, F.; Poncelet, P.; et al. Endothelial PTP1B Deletion Promotes VWF Exocytosis and Venous Thromboinflammation. *Circ. Res.* **2024**, *134*, E93–E111. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Drakeford, C.; Aguila, S.; Roche, F.; Hokamp, K.; Fazavana, J.; Cervantes, M.P.; Curtis, A.M.; Hawerkamp, H.C.; Dhimi, S.P.S.; Charles-Messance, H.; et al. von Willebrand factor links primary hemostasis to innate immunity. *Nat. Commun.* **2022**, *13*, 1–19. [\[CrossRef\]](#)
23. Lip, G.Y.H.; Blann, A. von Willebrand factor: A marker of endothelial dysfunction in vascular disorders? *Cardiovasc. Res.* **1997**, *34*, 255–265. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Gragnano, F.; Sperlongano, S.; Golia, E.; Natale, F.; Bianchi, R.; Crisci, M.; Fimiani, F.; Pariggiano, I.; Diana, V.; Carbone, A.; et al. The Role of von Willebrand Factor in Vascular Inflammation: From Pathogenesis to Targeted Therapy. *Mediat. Inflamm.* **2017**, *2017*, 5620314. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Mojiri, A.; Alavi, P.; Carrillo, M.A.L.; Nakhaei-Nejad, M.; Sergi, C.M.; Thebaud, B.; Aird, W.C.; Jahroudi, N. Endothelial cells of different organs exhibit heterogeneity in von Willebrand factor expression in response to hypoxia. *Atherosclerosis* **2019**, *282*, 1–10. [\[CrossRef\]](#)
26. Qian, J.; Fulton, D. Post-translational regulation of endothelial nitric oxide synthase in vascular endothelium. *Front. Physiol.* **2013**, *4*, 347. [\[CrossRef\]](#)
27. Böhm, F.; Pernow, J. The importance of endothelin-1 for vascular dysfunction in cardiovascular disease. *Cardiovasc. Res.* **2007**, *76*, 8–18. [\[CrossRef\]](#)
28. Xu, H.; Duan, J.; Tao, J.; Wang, W.; Wu, Y.; Dai, S.; Ren, J. Oxidized LDL Regulates Endothelin-1 and Oxidative Stress in Vascular Endothelial Cells: Role of Extracellular Regulated Kinase1/2 (ERK1/2). *Int. J. Drug Discov. Pharmacol.* **2023**, *2*, 18–29.
29. Niemann, B.; Rohrbach, S.; Catar, R.A.; Muller, G.; Barton, M.; Morawietz, H. Native and oxidized low-density lipoproteins stimulate endothelin- converting enzyme-1 expression in human endothelial cells. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 747–753. [\[CrossRef\]](#)
30. Secker, G.A.; Harvey, N.L. VEGFR signaling during lymphatic vascular development: From progenitor cells to functional vessels. *Dev. Dyn.* **2015**, *244*, 323–331. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Cesarman-Maus, G.; Hajjar, K.A. Molecular mechanisms of fibrinolysis. *Br. J. Haematol.* **2005**, *129*, 307–321. [\[CrossRef\]](#)
32. Hudson, N.E. Biophysical Mechanisms Mediating Fibrin Fiber Lysis. *BioMed Res. Int.* **2017**, *2017*, 2748340. [\[CrossRef\]](#)
33. Fukai, T.; Ushio-Fukai, M. Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases. *Antioxid. Redox Signal.* **2011**, *15*, 1583. [\[CrossRef\]](#)
34. Yang, J.; Shen, M.H. Polyethylene Glycol-Mediated Cell Fusion. *Methods Mol. Biol.* **2006**, *325*, 59–66. [\[PubMed\]](#)
35. Ishida, T.; Li, W.; Liu, Z.; Kiwada, H. Stimulatory effect of polyethylene glycol (PEG) on gene expression in mouse liver following hydrodynamics-based transfection. *J. Gene Med.* **2006**, *8*, 324–334. [\[CrossRef\]](#)
36. Weiss, S.N.; Legato, J.M.; Liu, Y.; Vaccaro, C.N.; Da Silva, R.P.; Miskiel, S.; Gilbert, G.V.; Hakonarson, H.; Fuller, D.A.; Buono, R.J. An analysis of differential gene expression in peripheral nerve and muscle utilizing RNA sequencing after polyethylene glycol nerve fusion in a rat sciatic nerve injury model. *PLoS ONE* **2024**, *19*, e0304773. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Spano, A.; Sciola, L. Polyploid cell dynamics and death before and after PEG-treatment of a NIH/3T3 derived culture: Vinblastine effects on the regulation of cell subpopulations heterogeneity. *Cell Div.* **2023**, *18*, 1–28. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Khovantseva, U.; Kiseleva, D.; Cherednichenko, V.; Chakal, D.; Breshenkov, D.; Markina, Y.; Ziganshin, R.; Charchyan, E.; Markin, A. The new perspective on understanding the mechanisms of cardiovascular diseases development. *Sci. Rep.* **2025**, *15*, 26596. [\[CrossRef\]](#)

39. Kawashima, Y.; Nagai, H.; Konno, R.; Ishikawa, M.; Nakajima, D.; Sato, H.; Nakamura, R.; Furuyashiki, T.; Ohara, O. Single-Shot 10K Proteome Approach: Over 10,000 Protein Identifications by Data-Independent Acquisition-Based Single-Shot Proteomics with Ion Mobility Spectrometry. *J. Proteome Res.* **2022**, *21*, 1418–1427. [[CrossRef](#)]
40. Deshmukh, A.S.; Cox, J.; Jensen, L.J.; Meissner, F.; Mann, M. Secretome Analysis of Lipid-Induced Insulin Resistance in Skeletal Muscle Cells by a Combined Experimental and Bioinformatics Workflow. *J. Proteome Res.* **2015**, *14*, 4885–4895. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.