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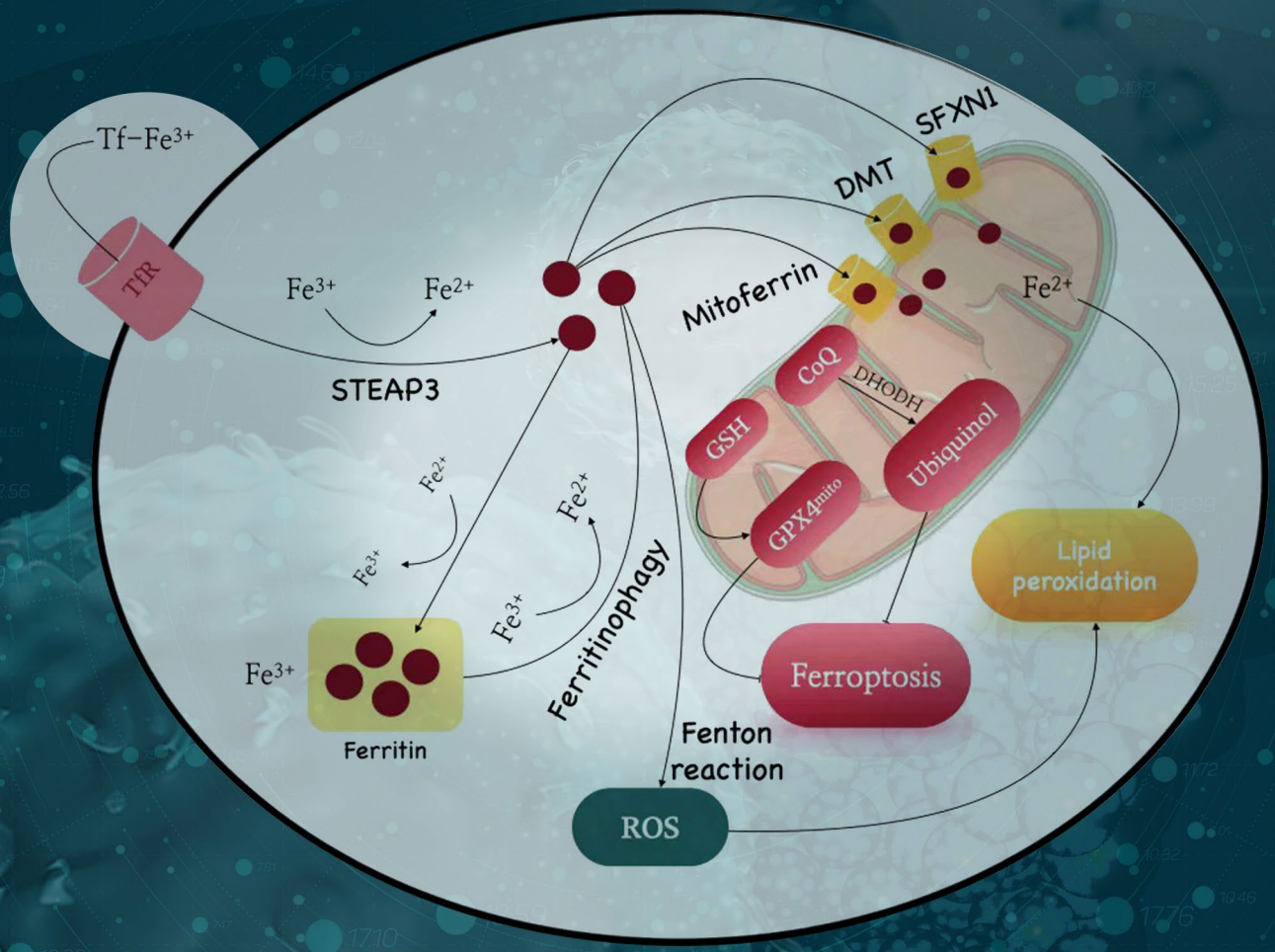

Mitochondrial involvement in ferroptotic cell death

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EDITORIAL

Omicron or no longer omicron: That is the question

Giuseppe Lippi*

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In an interesting article published in *Global Translational Medicine*, Bose *et al.*[1] attempted to compare the human immune response to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants Alpha (B.1.1.7), Delta (B.1.617.2), and Omicron (B.1.1.529). These valuable efforts contributed to a better understanding of the complex mechanism by which SARS-CoV-2 interacts with the human host over time, but their clinical significance must be balanced against the ongoing evolution of the viral genome. In their review of the literature, the authors used the generic term "Omicron" to group a number of subvariants. However, the original "Omicron" (B.1.1.529) strain has accumulated such an elevated number of mutations, since its first detection in November 2021, that the currently circulating strains share only a limited number of biological and immunological characteristics compared with their ancestor, and no longer justify the use of a generic name such as "Omicron."[2]

In a recent study, on the antigenicity, infectivity, cell-to-cell fusion properties, and spike protein processing of BA.2.87.1 and JN.1, Li *et al.*[3] reported that these two "Omicron" subvariants, which were still concomitantly circulating at the time of writing this letter, can exhibit dramatically heterogeneous biological behaviors, providing a clear example of how even two subvariants of the same strain display distinct behaviors. Notably, the authors discovered that compared to JN.1, BA.2.87.1 had a lower rate of immune escape from the sera of both coronavirus disease 2019 vaccine recipients and individuals with JN.1 breakthrough infection. Furthermore, BA.2.87.1 was found to have higher infectivity potential, cell-to-cell fusion activity, and spike protein cleavage efficiency than JN.1. These observations indicate a wide distinction of the biological characteristics between the two subvariants under the same "Omicron" clade.

The unpredictable evolutionary trajectory of SARS-CoV-2 variants poses significant challenges to develop clinically effective vaccines and therapies, such as antivirals and monoclonal antibodies. What has become clear with SARS-CoV-2 is that what was biologically plausible yesterday may not be today, as a new variant may have replaced the former. Aside from the critical importance of the continuous surveillance in detecting new biological properties and enhanced immune escape properties of new SARS-CoV-2 variants, I am persuaded that the use of Greek letters (such as Omicron) under which a large number of SARS-CoV-2 variants is included must be finally abandoned, since it is no longer biologically, clinically, or immunologically representative.

Conflict of interest

The author declares no conflicts of interest.

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REVIEW ARTICLE

Mitochondrial involvement in ferroptotic cell death

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Abstract

Ferroptosis is a regulated cell death pathophysiologically associated with the depletion of the antioxidant system due to iron overload, which results in excess lipid peroxidation. Mitochondria are crucial organelles known for their prominent involvement in various cellular metabolic activities and cell death processes. While our understanding of ferroptotic signaling pathways is advancing, further investigation into the intricate relationship between mitochondrial bio-process and this mode of cell death is necessary to identify effective biomedical therapeutic options targeting this organelle. However, the direct involvement of mitochondria in ferroptosis has remained a topic of debate due to the limited availability of concrete information to date. This review aims to elucidate the pathophysiological perspectives of mitochondria during ferroptotic cell death.

Keywords: Ferroptosis; Mitochondria; Cell death

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1. Introduction

Ferroptosis is a regulated form of cell death characterized by iron-dependent lipid peroxidation and oxidative damage, which eventually triggers the loss of membrane integrity and the release of molecules associated with cellular damage. Iron overload (alteration in iron homeostasis) plays a crucial role in generating reactive oxygen species (ROS) and promoting lipid peroxidation. The delicate balance of iron homeostasis, regulated by translational and transcriptional mechanisms, determines the susceptibility to ferroptosis, which leads to various pathologies, such as neurological disorders and ischemia-reperfusion injury.[1] A thorough understanding of the molecular mechanisms underlying iron metabolism during ferroptotic cell death may pave the way for effective therapeutic strategies for these diseases. Notably, iron chelators have demonstrated significant efficacy in preventing ferroptosis, providing novel therapeutic approaches for iron-related pathophysiological conditions.[2]

Ferroptosis is a type of iron-mediated, non-apoptotic regulated cell death. Mechanistically, iron accumulation, increased lipid peroxidation, and the inability to efficiently diminish lipid peroxidation are the main drivers of ferroptosis. While iron is essential for oxygen transport through hemoglobin and various metabolic pathways,

excessive accumulation of free redox-active iron (Fe^{2+}) triggers the overproduction of ROS through the Fenton reaction, which, in turn, leads to the production of lipid peroxides. Therefore, proper maintenance of iron homeostasis is important for cell survival. Extracellular ferric (Fe^{3+}) ions are internalized through transferrin receptor 1 (TfR1) and subsequently reduced into ferrous (Fe^{2+}) ions within late endosomes or lysosomes by the metalloreductase STEAP3. These Fe^{2+} are then transported into the cytosol by the divalent metal transporter (DMT1). Within the cytosol, Fe^{2+} can be exported from the cell by ferroportin (FPN1), incorporated into iron-containing proteins, or further converted to Fe^{3+} and stored within ferritin, contributing to the labile iron pool[3] (Figures 1 and 2).

Ferroptosis differs from other forms of cell death, including apoptosis, autophagy, necrosis, and pyroptosis, through its unique morphological, biochemical, and genetic characteristics. Its defining features include reduced mitochondrial volume, loss of mitochondrial cristae, condensed mitochondrial membrane density, and outer membrane rupture. Given the crucial role in iron and energy metabolism, mitochondria play an indispensable role in ferroptotic activities. Interference with important regulators of iron homeostasis, glutamine (Gln) metabolism, mitochondrial lipid metabolism, and other signaling pathways may influence ferroptotic sensitivity.[4] The intricate crosstalk between signals originating from various cellular organelles regulates a cell's ability to undergo ferroptosis. Compelling data suggests the involvement of numerous cellular organelles, such as the nucleus, mitochondria, endoplasmic reticulum, lysosomes, Golgi apparatus, and peroxisomes, in either triggering or inhibiting ferroptosis. This observation is noteworthy as these organelles are implicated in a number of disorders and may serve as targets for pharmaceutical therapies.[5]

Mitochondria are considered one of the most important subcellular organelles, playing critical roles in diverse cellular physiological activities. Most importantly, mitochondria are essential for energy production, cellular metabolism, and the regulation of cell death. However, the precise functions of these vital organelles before, during, and after the onset of ferroptosis remain unclear. The cellular respiratory system within mitochondria is responsible for generating ROS, which can lead to the formation of toxic metabolites[6] (Figure 2). Conversely, mitochondria possess a defense mechanism that aids in removing lipid peroxides to counteract ferroptosis. Recent research has shed light on the regulatory roles of mitochondria in ferroptosis. Understanding how mitochondria function during

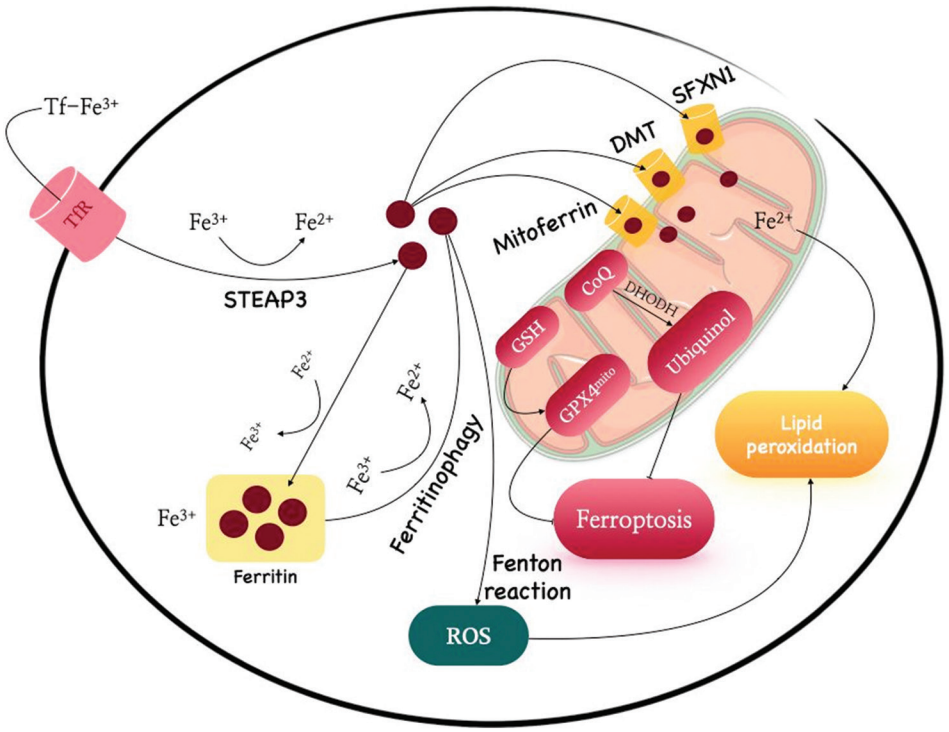

Figure 1. Iron ion uptake in the cell and mitochondria.
Abbreviations: CoQ: Ubiquinone; DHODH: Dihydroorotate dehydrogenase; DMT: Divalent metal transporter; GSH: Glutathione; GPX4: Glutathione peroxidase 4; ROS: Reactive oxygen species; SFXN1: Sideroflexin1; Tf: Transferrin; TfR: Transferrin receptor.

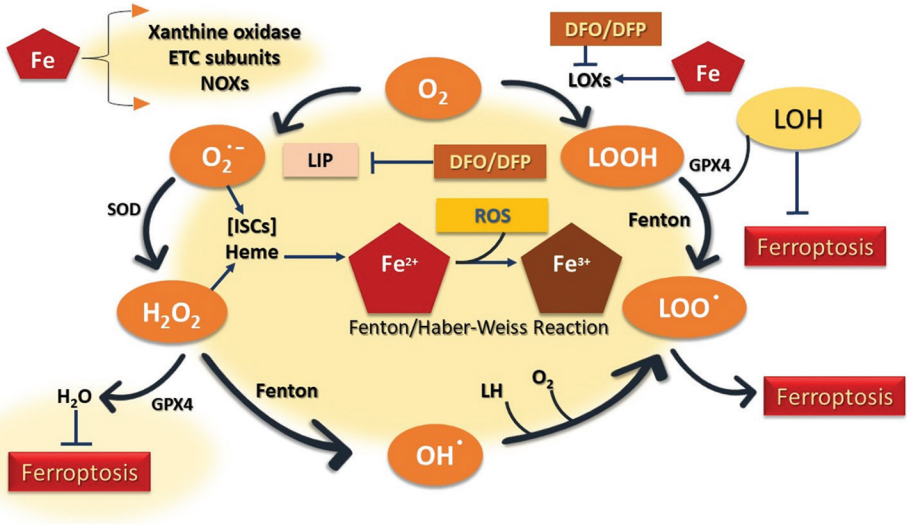

Figure 2. The oxidative stress generation during ferroptosis.
Abbreviations: DFO: Deferoxamine; DFP: Deferiprone; ETC: Electron transport chain; GPX4: Glutathione peroxidase 4; H_2O_2: Hydrogen peroxide; ISC: Iron-sulfur cluster; LH: Lipid; LIP: Labile iron pool; LOH: Lipid alcohols; LOO•: Lipid peroxyl radical; LOOH: Lipid hydroperoxide; LOXs: Lipoxygenases; $O_2^{\bullet-}$: Superoxide; NOXs: nicotinamide-adenine dinucleotide phosphate oxidases; OH•: Hydroxyl radicals; ROS: Reactive oxygen species; SOD: Superoxide dismutase.

ferroptosis has important ramifications for both basic cell biology and medicinal research. Mitochondrial electron transport chains (ETCs) drive proton motive force and adenosine triphosphate (ATP) synthesis, preventing AMP-activated protein kinase activation induced by energy stress and thereby promoting ferroptosis. Furthermore, electron leakage from ETC complexes I and III produces superoxide ($O_2^{\bullet-}$), which may promote polyunsaturated fatty acid peroxidation and subsequent ferroptosis. Glutaminolysis and the tricarboxylic acid (TCA) cycle in mitochondria further promote ferroptosis by driving ETC activities[7] (Figure 3). In addition to ATP production, mitochondria efficiently regulate cellular redox state and iron homeostasis, both of which have been proposed as mediators of ferroptotic signaling pathways. Prior studies regarding the role of mitochondria in ferroptotic cell death highlight the need for further study to achieve a clearer understanding of the pathophysiology of ferroptosis.[8] Given that mitochondria are known to function during oxidative stress and other cell death processes, exploring their potential functionality during iron overload-induced ferroptotic cell death is a logical next step.

2. Relationship between mitochondria and ferroptosis

Studies demonstrating that mitochondrial malfunction and damage exacerbate oxidative stress, subsequently causing ferroptosis, suggest a strong relationship between ferroptosis and mitochondrial function. The development of numerous illnesses is intimately linked to changes in the structure and function of mitochondria, which are essential for maintaining cellular homeostasis. As extremely active organelles, mitochondria are regulated by a number of mechanisms to ensure stability. While essential mechanisms, including mitophagy, mitochondrial fusion, and fission, play a major role in dynamically maintaining mitochondrial homeostasis, dysregulation of these processes can occur. Ferroptosis is closely associated with mitophagy, as well as the mitotic fission and fusion of mitochondria. Investigating the dynamic regulation of mitochondrial activities during ferroptosis is crucial for a better understanding of illness development. To advance a thorough comprehension of ferroptosis and offer a comparable reference for the treatment of associated disorders, we have methodically summarized alterations in ferroptosis, mitochondrial fission and fusion, and mitophagy in this article.[9]

Numerous clinical diseases, such as ischemic tissue damage, infection, dementia, and cancer, are linked to ferroptosis, a mode of cell death characterized by iron-dependent lipid peroxidation. The cellular machinery governing ferroptosis integrates multiple pro-survival or pro-death signals from subcellular organelles and then "decides" whether to initiate this lethal process. Various organelles, such as the nucleus, lysosomes, endoplasmic reticulum, lipid droplets, peroxisomes, Golgi apparatus, and mitochondria, play crucial roles in the initiation or prevention of ferroptosis.[5]

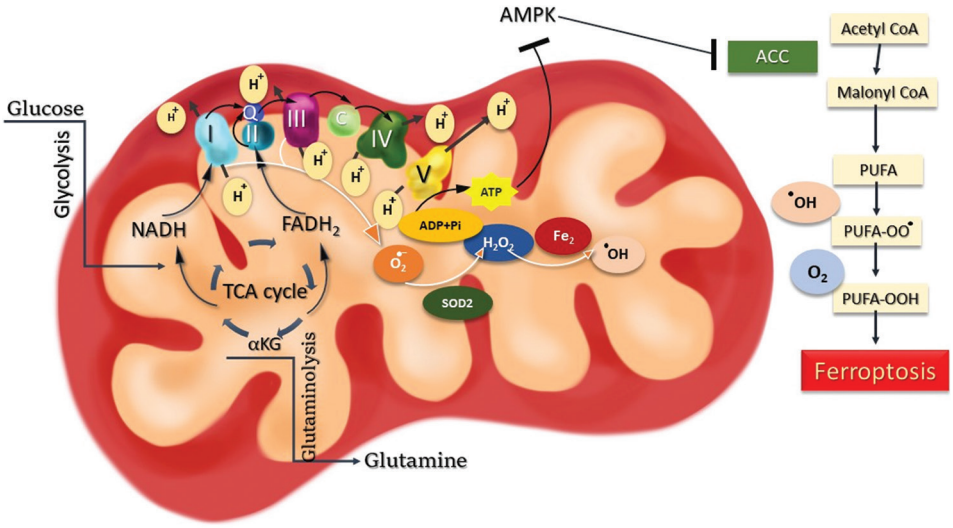

Figure 3. The crosstalk of cellular bioenergetic pathway in mitochondria. Figure adapted with modification from Gan.[7]
Abbreviations: αKG: Alpha-ketoglutarate; ACC: Acetyl-CoA carboxylase; ADP: Adenosine diphosphate; AMPK: AMP-activated protein kinase; ATP: Adenosine triphosphate; C: Cytochrome C; CoA: Coenzyme; FADH2: Flavin adenine dinucleotide; H_2O_2: Hydrogen peroxide; NADH: Reduced nicotinamide adenine dinucleotide; OH•: Hydroxyl radicals; Pi: Inorganic phosphate; PUFA: Polyunsaturated fatty acid; Q: Ubiquinol; SOD2: Superoxide dismutase 2; TCA: Tricarboxylic acid.

Compared to other controlled cell death processes, including apoptosis, autophagy, necrosis, and pyroptosis, ferroptosis, an iron-dependent cell death process, clearly differs in terms of morphology, biochemistry, and genetics. Characteristics such as condensed mitochondrial membrane density, reduced volume compared to normal mitochondria, and the presence of decreased or absent mitochondrial cristae and ruptured outer membranes are prominent features of ferroptosis. As the primary organelle in iron utilization, as well as in catabolic and anabolic pathways, mitochondria play a major role in iron metabolism, substance metabolism, and energy metabolism. Ferroptotic sensitivity is affected by interference from important regulators of iron homeostasis (ferritin, mitoferrin1/2, and NEET proteins), mitochondrial lipid metabolism (acyl-coenzyme [CoA] syntheses family member 2 and citrate synthase [CS]), Gln metabolism, and other signaling pathways. Targeted stimulation of ferroptosis emerges as a potential treatment approach for numerous oxidative stress conditions, such as neurodegenerative disorders, ischemia-reperfusion injury, and traumatic spinal cord injury. Nonetheless, the relevance of ferroptosis and mitochondria remains subject to debate. In this article, we methodically clarify how the morphological traits and metabolic control of mitochondria relate to the regulation of ferroptosis (Figure 4).[4]

The role of hydrogen peroxide (H_2O_2) in triggering ferroptosis is vital. Studies have classified ferroptosis as a form of H_2O_2-induced cell death. It has been observed that cells lacking mitochondrial DNA exhibit elevated levels of lipid peroxidation levels and heightened sensitivity to H_2O_2. In addition, aquaporin proteins (AQP 3, 5, and 8) have been found to interact with nicotinamide-adenine dinucleotide phosphate oxidase 2, regulating extracellular H_2O_2 permeability and thus influencing ferroptosis. By reducing Fe^{2+} levels and upregulating the mitochondrial quality control protein prohibitin 2, which subsequently decreases AQP expression, mitochondrial transfer into these cells has been shown to reduce their susceptibility to H_2O_2-induced cytotoxicity. These results imply that H_2O_2 therapy holds potential as an effective cancer treatment approach by modulating these pathways.[10]

The availability of cysteine, obtained through system Xc^- or the trans-sulfuration route, as well as the production of glutathione (GSH) and the appropriate operation of GPX4, are necessary for the prevention of iron-dependent lipid peroxidation and ferroptosis. Ferroptosis has been linked to neurodegeneration and ischemia/reperfusion injuries in animal models, yet its exact function in normal physiological processes remains unclear. Recent research suggests that interferon-gamma generated by $CD8^+$ T cells and the tumor suppressors p53 and BAP1 regulate system Xc^-.[11]

Reduced GSH activity and impaired GSH peroxidase-4 (GPX4) defense serve as two biochemical markers of ferroptosis, indicating a compromised antioxidant system. Recent research has demonstrated that oxidative glutamate

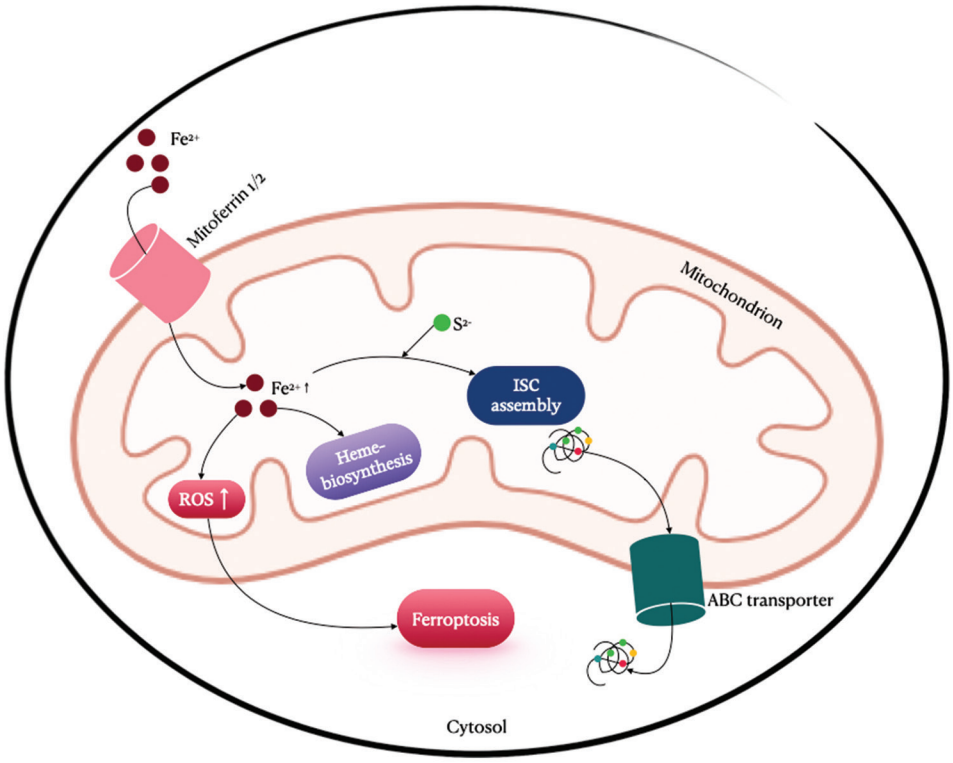

Figure 4. The overall pathophysiology of mitochondrial iron regulation during ferroptosis.
Abbreviations: ABC: ATP-binding cassette; ISC: Iron-sulfur cluster.

toxicity, GPX4 depletion, and ferroptosis models all result in mitochondrial injury. RAS-selective lethal (RSL3) induces ferroptosis in neuronal cells and fibroblasts. Protecting mitochondrial integrity and function using different biochemical methods appears to mitigate RSL3 toxicity-induced ferroptosis, implying that targeting mitochondria could be a promising therapeutic strategy in conditions of extreme oxidative stress and cell death.[12]

Ferroptosis is heavily influenced by mitochondria and nuclear factor erythroid 2-related factor 2 (NRF2), given their well-established functions in the oxidative stress pathway. Researchers have observed that a high-iron diet increases liver iron levels, causing oxidative stress, lipid peroxidation, and reduced GSH. In addition, iron overload upregulates the expression of acyl-CoA synthetase long-chain family member 4 and downregulates the expression of GPX4 and cystine-glutamate antiporter (SLC7A11), indicative of ferroptosis. Moreover, iron excess induces lipid peroxidation, decreases mitochondrial membrane potential (MMP), and increases ROS production. Treatment with an iron chelator reduces lipid peroxidation and ROS, demonstrating a clear correlation between iron excess and ROS levels. The reduction of mitochondrial oxidative stress attenuates ferroptosis. A previous study has demonstrated that iron-induced ferroptosis prevents NRF2 binding to antioxidant response elements (AREs) within the promoters of the *gpx4* and *slc7a11* genes, resulting in transcriptional suppression in HEK293T cells. Overall, the study has elucidated a clear link between ferroptosis, the NRF2-ARE pathway, and mitochondrial oxidative stress.[13]

In contrast to GPX4-induced ferroptosis, mitochondria are important in cysteine-deprivation-induced ferroptosis. Cysteine deficiency leads to the accumulation of lipid peroxides and hyperpolarization of the MMP. Inhibition of the mitochondrial ETC or TCA cycle can halt ferroptosis, lipid peroxide accumulation, and hyperpolarization. Similarly, blocking glutaminolysis can be counteracted by providing intermediates of the downstream TCA cycle. Loss of fumarate hydratase, a tumor suppressor and TCA cycle component, confers resistance to cysteine-deprivation-induced ferroptosis. These results highlight the pivotal role of mitochondria in cysteine deprivation-induced ferroptosis and its potential for tumor suppression.[14]

Studies have revealed that major indicators involved in ferroptosis, such as nicotinamide adenine dinucleotide phosphate hydrogen, GSH, and ROS, are regulated by cellular energy metabolism processes such as glycolysis, pentose phosphate pathway (PPP), and the TCA cycle.

Tumor cells, in particular, may trigger adaptive metabolic responses, including upregulation of the PPP and glycolysis, to defend against ferroptosis. Exploiting these metabolic vulnerabilities, such as changes in glucose metabolism and the reliance on Gln for metabolic compensation, may offer new therapeutic avenues for inducing ferroptosis and disrupting redox homeostasis, thereby providing novel therapeutic options for tumor treatments.[15]

The mitochondria serve as the primary organelle in both anabolic and catabolic processes. The breakdown of Gln, which is crucial for ferroptosis, is facilitated by enzymes such as glutamate dehydrogenase, glutamate oxaloacetate transaminase 2, glutaminase, and glutamate pyruvate transaminase. This process produces substrates for lipid synthesis and the TCA cycle. During ferroptosis, alpha-ketoglutarate and subsequent TCA metabolites can serve as substitutes for Gln. ACSF2 and CS regulate fatty acid activation and synthesis, respectively, providing lipid precursors necessary for lipid oxidation. The cysteine/glutamate antiporter system Xc^-, which imports cysteine for the production of GSH, is restrained by ferroptosis inducers. Ferroptotic cell death-causing lipid peroxides are eliminated by GPX4 through GSH supplementation. The amount of iron present in the cytosol is linked to iron absorption, storage, utilization, and efflux from cells, processes controlled by iron regulatory proteins such as ferritin, FPN1, and TfR1. Mitochondrial iron absorption is also mediated by voltage-dependent anion channels (VDACs). Through the mitochondrial iron transporter mitoferrin, cytosolic iron enters the mitochondrial matrix, where it is primarily used to produce heme and Fe/S clusters, some of which are deposited in mitochondrial ferritin. Lipid peroxidation and ROS accumulation are induced by increased labile iron buildup in the mitochondria. The mitochondrial inner membrane ATP-binding cassette (ABC) transporter ABCB7 exports the Fe/S-cluster complex produced by the mitochondrial ISC (Fe/S cluster assembly) system into the cytosol, where it matures further in the cytosolic Fe/S protein assembly (CIA) system. NEET proteins, localized to the outer mitochondrial membrane, mediate the import and export of iron and sulfur ions, facilitating the mobilization of 2Fe-2S clusters to cytosolic apo-acceptor proteins. In addition, the formation of mitochondrial Fe/S proteins uses the Fe/S cluster.[4]

In a study involving SK-Hep1 ρ0 cells,[16] there was an increase in the expression of mitochondrial-type GPX4 (mGPX4), while other types of GPX4 remained unchanged. SK-Hep1 ρ0 cells exhibited resistance to ferroptosis induced by erastin, which blocks the cystine-glutamate exchanger (xCT) channel, likely due to high mGPx4 expression. Conversely, SK-Hep1 ρ0 cells were susceptible to RSL3-induced ferroptosis, which suppresses GPx4. In SK-Hep1 ρ+ cells or cells treated with RSL3 and erastin, there was an accumulation of cellular ROS and oxidized lipids, whereas erastin-treated SK-Hep1 ρ0 cells did not exhibit such accumulation. The action of RSL3 and erastin on xCT on the plasma membrane led to increased ROS and lipids peroxidation in SK-Hep1 ρ+ cells. The inhibition of SK-Hep1 ρ+ cell death by erastin or a high dosage of RSL3 was observed on mitochondrial ROS quenching, indicating a crucial function of mitochondrial ROS in ferroptosis. Compared to DecylQ, a non-targeting equivalent, the concentration of MitoQ, a mitochondrial ROS quencher, required to reduce ferroptosis induced by erastin or RSL3, was more than 20 times lower. Furthermore, a VDAC inhibitor significantly reduced the accumulation of mitochondria ROS, total peroxidized lipids, and mitochondrial peroxidized lipids, along with the ferroptosis of SK-Hep1 ρ+ cells induced by erastin or RSL3. This finding supports the involvement of mitochondrial events in ferroptotic death and the role of VDAC in the mitochondrial steps of ferroptosis induced by erastin or RSL3. In addition, mitochondrial ROS quenchers prevented sorafenib-induced mitochondrial ROS and mitochondrial peroxidized lipid accumulation while also suppressing the sorafenib-induced ferroptosis of SK-Hep1 ρ+ cells. According to these findings, it is suggested that SK-Hep1 ρ0 cells may be immune to ferroptosis due to overexpression of mGPx4, and mitochondrial processes may play a decisive role in determining the ultimate destiny of the cell.[17]

Due to their altered morphology, mitochondria are recognized as essential players in both the initiation and execution of ferroptosis. Mitochondrial involvement significantly influences ferroptosis in a number of pathological conditions, including cancers, heart diseases, and neurological disorders. Further exploration of the interplay between ferroptosis and mitochondria in conditions such as diabetes, liver diseases, and renal disorders is imperative. Future studies should focus on investigating mitochondria-targeted approaches as potential therapeutic therapies for conditions associated with ferroptosis.[18]

Eukaryotic cells require metabolic flexibility to respond to changes in their environment. Mammalian cells exhibit remarkable flexibility in maintaining cellular energy homeostasis across diverse circumstances, including neurodegenerative diseases, due to their capacity to transition from mitochondrial respiration to aerobic glycolysis. In neurodegenerative research, ferroptosis, a form of cell death induced by redox imbalance, is

becoming more and more prominent. Recent research has revealed that neuronal cells are shielded against oxidative death through the activation of small-conductance calcium-activated K^+ (SK) channels, which also regulate mitochondrial respiration. Investigations are underway to determine whether SK channel activation causes a change in glycolysis, rendering neuronal cells more resistant to ferroptosis. The findings indicate that activation of the SK channel increases lactate production and glycolysis while mildly decreasing mitochondrial complex I (CI) activity. Moreover, SK channel activation prevents neuronal cells from undergoing ferroptosis by scavenging mitochondrial ROS and blocking glycolysis. Furthermore, experiments involving *Caenorhabditis elegans*, a model worm, demonstrated that SK channel activation increases survival rates in the presence of mitochondrial toxins. These results emphasize the metabolic pathways facilitated by SK channel activation, supporting neuronal resistance to ferroptosis *in vitro* and extending lifespan *in vivo*.[16]

A well-known protein kinase, glycogen synthase kinase-3 beta (GSK-3β), has recently been discovered as a positive regulator for ferroptosis. Resistance to ferroptosis is enhanced upon inhibition of GSK-3β, either through genetic knockdown or treatment with the medication LY2090314. GSK-3β disrupts iron homeostasis by inhibiting the expression of DMT1, ferritin heavy chain/light chain, and other iron metabolic components, thereby reducing the intracellular pool of labile free iron. These results demonstrate that modulating GSK-3β activity to influence ferroptotic sensitivity represents a promising strategy for cancer therapy.[19]

In melanoma cells, inhibition of mitochondrial CI by BAY 87–2243 results in both inhibition of cancer growth and induction of cell death. Treatment with BAY leads to depolarization of the MMP, elevated levels of ROS and lipid peroxidation, and reduction of GSH levels. These effects are accompanied by mitophagy, autophagosome production, and enhanced opening of the mitochondrial permeability transition pore (mPTP). The cell death induced by BAY is not attributed to glucose deprivation and can be prevented by mPTP inhibitors and antioxidants. Knockdown of TRAP1 increases cell death, whereas its overexpression decreases ROS levels and prevents cell death. Knockdown of Atg5 prevents the production of autophagosomes, an increase in ROS levels, and cell death. PINK1 knockdown prevents cell death, mitochondrial depolarization, mitophagy, and an increase in ROS. *Drp1* knockdown prevents BAY-induced cell death and promotes mitochondrial filamentation. Pancaspase inhibitors have no effect on BAY-induced cell death, whereas necroptosis inhibitors and necroptosis protein knockdown do. Lipid peroxidation, ROS accumulation, and cell death induced by BAY are all reduced by the ferroptosis inhibitor and GPX4 overexpression. Conversely, inhibition of GPX4 increases cell death. According to the suggested sequence of events, CI inhibition leads to mPTP opening and depolarization, triggering autophagy, an increase in ROS, and ultimately a combination of necroptotic/ferroptotic cell death.[20]

Through genome-wide CRISPR screening, scientists have demonstrated that mitochondrial calcium uptake 1 (MICU1) plays a crucial role in the production of lipid peroxide and subsequent ferroptosis during cold stress. MICU1 acts as a regulator in this process by controlling the mitochondrial calcium uniporter. Under cold stress conditions, MICU1 promotes an increase in mitochondrial calcium levels, hyperpolarization of the MMP, and the ensuing lipid peroxidation. These results demonstrate the role of the MICU1-dependent mitochondrial calcium homeostasis-MMP hyperpolarization axis in cold stress-induced lipid peroxidation and ferroptosis.[21]

The process of brain cell death is a complex pathologic event that can be triggered by the oxidative stressor tert-butylhydroperoxide (t-BHP). Researchers have discovered that t-BHP induces ferroptosis, a form of oxidative stress-related cell death, based on the presence of ferroptosis indicators. During the dying process, various pathophysiological changes occur, primarily related to mitochondrial dysfunction, including reduced synthesis of ATP, membrane potential, and increased production of ROS within the mitochondria. The ferroptosis inhibitor ferrostatin-1 has been observed to ameliorate these mitochondrial defects. Furthermore, ferroptosis and mitochondrial malfunction have been linked to upstream processes involving the activation of the JNK1/2 and ERK1/2 pathways. These findings suggest that targeting oxidative stress pathways could serve as a viable strategy for protecting against neurodegenerative diseases.[22]

A novel medication class known as Abivertinib (AC) is recognized for its ability to suppress the function of epidermal growth factor receptors implicated in tumorigenesis. It is shown that AC induces cancer cells to undergo either ferroptosis or apoptosis, two different forms of cell death. In addition, it has been demonstrated that AC induces the accumulation of iron and ROS in a number of cancer cell lines, which results in cell death. Moreover, AC triggers the accumulation of toxic lipid ROS and reduces the production of certain proteins involved in cellular defense, compromising the integrity of the cell membrane. Furthermore, AC activates caspase-3 and other apoptosis-related enzymes, suggesting ROS-dependent apoptosis. The processes of ferroptosis and apoptosis induced by AC are significantly influenced by mitochondria. Moreover,

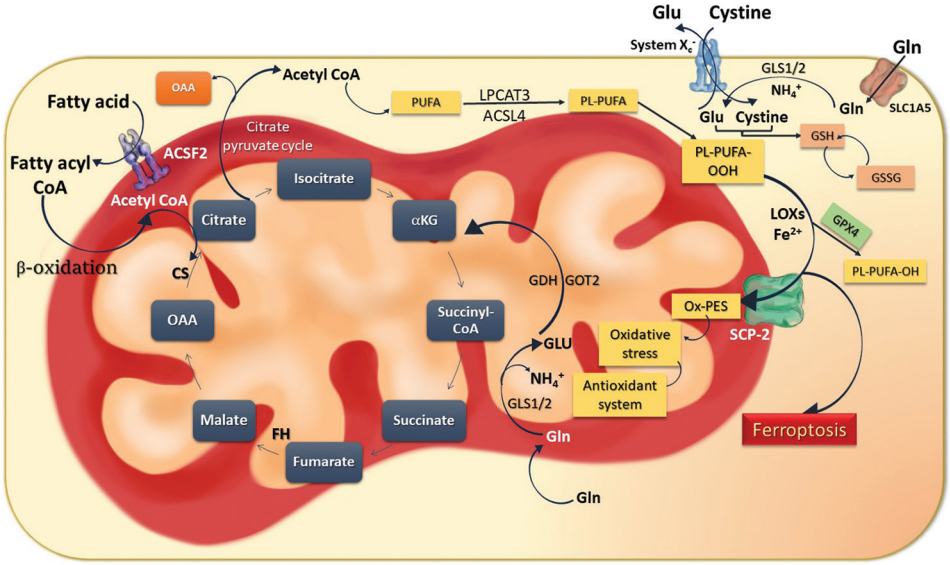

Figure 5. Overview of the mitochondrial metabolic regulation during ferroptotic cell death. Modified from Wang *et al.*, 2020[4]

upstream regulators such as Bax and Bim proteins play crucial roles in AC-induced ferroptosis and apoptosis. In conclusion, AC exerts its anticancer activity primarily through ferroptosis and apoptosis, regulated by the mitochondrial pathways involving Bax and Bim.[23]

Furthermore, along with the increase of endoplasmic reticulum stress and the inhibition of certain transporters, the process entails the activation of particular channels and kinases. Ferroptosis is associated with the accumulation of ROS and lipid peroxidation products resulting from iron metabolism. Pharmacological inhibition of ferroptosis can be achieved through lipid peroxidation inhibitors and iron chelators. The process of ferroptosis is regulated by a number of proteins that can either promote or inhibit it. Dysregulated ferroptosis has been implicated in numerous physiological and pathological processes, including neurotoxicity, cancer cell death, and damage to the kidneys and liver.[24]

Ferroptosis regulation is influenced by various metabolic factors, including organelle crosstalk. In human pancreatic cancer cells, the endoplasmic reticulum protein STING1 plays a pivotal role in facilitating ferroptosis. STING1 promotes mitochondrial fusion, a process dependent on MFN1/2, leading to the generation of ROS and lipid peroxidation. Depletion of STING1 or MFN1/2 reduces the likelihood of ferroptosis in pancreatic cancer cells. This biological process exemplifies the significance of mitochondrial fusion and carries important therapeutic implications.[25]

Different features are present in ferroptosis at the morphological, genetic, and biochemical levels. Robust correlations have been observed between dysregulated ferroptosis and both degenerative illnesses and organ damage in humans. Moreover, specifically promoting ferroptosis shows promise as a therapeutic approach for malignancies resistant to existing treatments. Mitochondria, the cellular powerhouse, are essential for regulating several forms of cell death, including ferroptosis. Recent research indicates a connection between ferroptosis and impaired mitochondrial morphology and function. Current paradigms in this field suggest that a significant amount of research is required to critically assess the complex control of ferroptosis by mitochondria, which will shed light on its potential molecular processes. In addition, the exploration of therapies targeting mitochondria holds potential as cutting-edge biomedical strategies for addressing ferroptosis-related disorders.[26]

3. Conclusion

Mitochondria are crucial organelles for regulating cell death pathways. Ferroptosis, a relatively newly discovered form of programmed cell death, has garnered significant attention in contemporary biomedical research, with researchers worldwide working to elucidate the pathways related to the pathophysiology of this iron-dependent cell death. To gain a comprehensive understanding of the cellular pathophysiological aspects of ferroptosis, it is necessary to concentrate on the involvements of subcellular organelles, precisely mitochondria. Our article explores the potential role of mitochondrial connections in this regard, and we anticipate that further research will be necessary to unravel the intricate pathways associated with mitochondria in relation to ferroptosis (Figure 5).

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REVIEW ARTICLE

Effects of flavonoids on vascular activity

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Abstract

Flavonoids, encompassing various polyphenolic compounds found in plants, exert significant effects on vascular function. Particularly notable is their role in inducing vasodilation, a process crucial for regulating blood pressure (BP) and enhancing cardiovascular health. Through their vasodilatory properties, flavonoids contribute to improved blood flow and endothelial function. In addition, flavonoids demonstrate antiplatelet effects, which play a vital role in preventing abnormal blood clot formation and reducing the risk of thrombotic events. By inhibiting platelet aggregation, flavonoids help maintain vascular integrity and mitigate the likelihood of cardiovascular complications. In addition to vasodilation and antiplatelet effects, flavonoids exhibit potential benefits in managing hypertension. Studies suggest that flavonoids can help regulate BP by promoting vasorelaxation and modulating endothelial function. These mechanisms contribute to the overall maintenance of cardiovascular homeostasis and may offer therapeutic avenues for hypertension management. This review aims to comprehensively explore the multifaceted effects of flavonoids on vascular function, with an emphasis on their vasodilatory properties, antiplatelet effects, and potential implications for hypertension management within the existing literature.

Keywords: Atherosclerosis; Flavonoid; Hypertension; Vascular functions

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1. Introduction

Flavonoids, a diverse family of approximately 5000 hydroxylated polyphenolic compounds, play a pivotal role in plants by aiding in resistance to environmental challenges and regulating cell growth.[1] These compounds belong to the category of plant secondary metabolites characterized by an aromatic ring with at least one hydroxyl group. The plant kingdom boasts over 8000 naturally occurring phenolic compounds, as identified in previous studies.[1,2]

The acetic acid and shikimic acid routes are used in the biosynthesis of these low molecular weight polyphenolic chemicals. Flavonoids are synthesized when three malonyl-CoA units (C-2) and one p-coumaric acid unit (C-9) condense. Structurally, flavonoids are distinguished by a basic core (C6-C3-C6) consisting of two benzene rings joined by a three-carbon unit, usually a pyrene ring containing oxygen. Some of their structural characteristics include a 2-phenyl chromone skeleton with different substitution patterns in the A-ring (typically a phloroglucinol or resorcinol hydroxylation

pattern) and the B-ring (generally catechol, pyrogalol, or 4'-hydroxylated).[3]

The oxidation of the C-ring results in the production of several types of flavonoids, such as flavans, flavanones, flavones, and flavanols, as well as catechins, anthocyanidins, and isoflavones. Flavonoids, also known as polyphenolic compounds, are classified into six types: flavonols, flavanones, flavanols, flavones, anthocyanidins, and isoflavonoids. Each type undergoes distinct metabolic processes within the human body.[2,4]

Flavonoids demonstrate substantial antioxidant activity, contributing to the body's defense against toxins. Their potent antioxidant properties, crucial for protecting plants from adverse environmental conditions, have attracted considerable scientific attention.[5] These compounds have been extensively investigated in epidemiological and experimental studies for their anti-inflammatory,[6] immunomodulatory,[7] and potent anticancer properties both *in vitro* and *in vivo*.[8]

Flavonoids play roles in diverse biological activities across animals, bacteria, and plants. In plants, they contribute to the color and fragrance of flowers, aid in attracting seed-dispersing agents in fruits, act as a defense against oxidative stress, and function as ultraviolet filters. These polyphenolic compounds are found in various plants, such as roses, and offer several health benefits, such as antimicrobial, antihypertensive, and bronchodilatory effects, making flavonoids potentially valuable for combating microorganisms, managing blood pressure (BP), and easing respiratory discomfort.[9-12] In addition, flavonoids safeguard plants from environmental stresses, serving as signaling agents, detoxifiers, and defensive molecules against pathogens.[13] Despite the considerable diversity among flavonoids, their low bioavailability and biological activities, such as vasodilation, anti-atherogenic modulation of cell signaling, regulation of gene expression related to disease development, reduction of hypertension, and prevention of platelet aggregation in humans, are contingent on their chemical nature. Fruits, nuts, seeds, vegetables, and beverages such as coffee, wine, and tea are rich sources of flavonoids, exhibiting significant antioxidant activity.[14] Flavonoids hold substantial therapeutic value in conditions such as Alzheimer's disease, atherosclerosis, and cancer.[15]

Flavonoids stand as major antioxidants in the human diet, with intake levels surpassing those of Vitamin C by 10 times and Vitamin E/carotenoids by 20 times. Their antioxidant, anticarcinogenic, anti-inflammatory, and antimutagenic actions have led to their incorporation in cosmetic, medicinal, nutritional, and pharmaceutical applications.[16] The qualitative and quantitative variations in flavonoids across plants are influenced by factors such as species, age, geographical distribution, cultivation method, plant part used, and storage conditions. In addition, abiotic factors such as sunlight exposure, soil type, precipitation, and the number of fruits per tree play a role in determining flavonoid content in plants.[1]

Over the last decade, flavonoids have garnered increased attention for their potential health benefits. Given their heterogeneous structure, studies investigating flavonoids are usually both long-term and complex. Numerous studies suggest that dietary flavonoids may serve as beneficial adjuncts in chronic inflammatory diseases.[17,18] Common flavonoid-rich foods, including vegetables and fruits, are extensively integrated into daily diets. For instance, apples, berries, grapes, kale, onions, and tomatoes are rich in flavones. In addition, celery, chamomile, mint, red peppers, parsley, citrus fruit peels, and beverages like red wine and tea contain abundant flavones. These flavones play a pivotal role in the vasodilation process, which involves the relaxation of smooth muscles in the walls of arteries and veins. This relaxation allows blood vessels to expand, facilitating increased blood flow and contributing to the reduction of BP.[19] The modulation of systemic vascular resistance and the promotion of increased blood flow are crucial components of vasodilation. The present review extensively discusses the role of flavonoids in vascular activity.

2. Sources and bioavailability of flavonoids

Polyphenolic compounds, including flavonoids, play a crucial role in plant flavor and coloration. They serve various essential functions in plants, including auxin transport, growth, pollinator attraction, reproduction, seed dispersal, protection against abiotic and biotic stresses, and resistance to predators.[20] Flavonoids are abundant in many vegetables (onions and lettuce), fruits (apples, limes, and cherries), grains, legumes, and tea; they are even present in processed foods such as wine and dark chocolate.[14] The flavonoid content varies depending on the source, species, sunlight exposure, season of collection, and other factors.[21] Physiologically, humans do not synthesize flavonoids, relying on plant sources for their intake. In humans, the small intestine absorbs flavonoids, which then undergo conjugation with glucuronic acid, sulfate, or O-methylation. This process varies significantly among all classes of flavonoids, but overall, the absorption rate remains high when flavonoids are consumed as part of integrated food.[22]

2.1. Flavones

Flavones comprise a closed pyran ring with a ketone at position 4, and positions 2 and 3 are linked by a double

bond.[23] Flavones have a ketone at position 4 of the closed pyran ring and a double bond between positions 2 and 3. These compounds are present in various foods, including celery, mint, red peppers, garlic, parsley, and chamomile flowers. Such sources of flavones are abundant in apigenin, luteolin, and tangeritin.[24] In addition, the peel of citrus fruits contains flavones (polymethoxylated flavones), such as nobiletin, tangeretin, and sinensetin.[25] Luteolin has been shown to decrease BP in hypertensive rats by reducing the proliferation of angiotensin I-induced vascular smooth muscle cells (VSMCs), enhancing vasodilation in the aorta, and inhibiting cAMP-specific phosphodiesterase, leading to the accumulation of cAMP.[26] Luteolin activates cAMP, subsequently triggering protein kinase A. Moreover, nitric oxide (NO) synthase becomes activated, elevating NO levels in endothelial cells. The accumulated NO promotes vascular relaxation through potassium (K^+) and calcium (Ca^{2+}) channels.[27]

2.2. Flavonols

Flavonols stand out among all flavonoids due to the presence of the ketone group, acting as precursors of proanthocyanins. These compounds feature a hydroxyl group at position 3 of the closed pyran ring, which can undergo glycosylation.[28] The most commonly encountered flavonols include quercetin, kaempferol, fisetin, and myricetin, abundant in foods such as broccoli, beans, kale, onion, lettuce, tea, tomatoes, apples, berries, strawberries, grapes, and wine.[29]

Quercetin, a prominent flavonol, is recognized for its antihypertensive effects. On consumption, quercetin enhances endothelial function and regulates the renin-angiotensin-aldosterone system (RAAS).[30] It induces vasodilation in the kidneys and lowers BP levels in diabetic patients. Studies have demonstrated its efficacy in addressing hypertension, hyperinsulinemia, and dyslipidemia, as well as inducing anti-inflammatory effects in vascular adipose tissue, leading to reduced body weight in obese Wistar rats.[31] Quercetin also provides relief from oxidative stress in organs such as the heart and kidneys.[32] Its antihypertensive properties are attributed to the release of NO in endothelial cells.[33]

2.3. Flavan-3-ols

In flavan-3-ols, the hydroxyl group at position 3 of the closed pyran ring is bonded, and the double bond is absent at positions 2 and 3. This category includes catechin, epicatechin, gallocatechin, and oligomers.[34] Catechins, found in various plants such as apples, apricots, cocoa, pears, and tea in the aglycone form, represent the monomeric form of flavanols.[35] Catechins exhibit positive effects on vascular functions, contributing to a cardioprotective impact, and have been shown to reduce both diastolic and systolic BP.[36]

Another type of catechin, epigallocatechin-3-gallate, is highly prevalent in green tea, serving as an ester of gallic acid and epigallocatechin. This compound exerts diverse effects on various physiological and pathological processes in humans, demonstrating bioactivities such as anti-atherogenic action, antioxidant properties, and anti-inflammatory effects.[34] Epicatechin, another flavan-3-ol flavonoid, is recognized for its antihypertensive action. The consumption of an epicatechin-supplemented diet has been linked to reductions in both systolic and diastolic BP, along with a decrease in myocardial tissue rigidity in rats with hypertrophic cardiomyopathy.[36,37]

2.4. Flavanones

Flavanones, alternatively known as dihydroflavones, exhibit a distinctive structural feature—a saturated closed pyran ring. Key flavanones in this class include naringenin and hesperetin, prominently found in citrus fruit peels. Renowned for their antioxidant properties, these compounds, as reviewed in Barreca *et al.*,[38] effectively block the activity of free radicals.[38] Hesperetin, a dietary flavanone, is notably abundant in lemons and sweet oranges. Rapid absorption from the intestine facilitates the generation of metabolites that demonstrate an antihypertensive effect. The mechanism involves G-hesperidin under hypertension, leading to lowered BP. In addition, the antioxidant nature of hesperetin contributes to increased nitric acid content and reduced Ca^{2+} levels, promoting the relaxation of vascular smooth muscles.[39]

Naringenin, found in grapefruit and certain herbs, has been subject to numerous studies investigating its beneficial effects.[40] A naringin-supplemented diet has shown promise in the treatment of hypertension, diabetes, antiviral and antibacterial activities, anti-inflammatory responses, antiadipogenic effects, and the management of the metabolic syndrome. Notably, naringenin is recognized for its ability to reduce BP, protect against endothelial dysfunction, and modulate NO levels.[41]

2.5. Anthocyanidins

Anthocyanidins, characterized by their water-soluble nature, stand as pigments unique among flavonoids, predominantly contributing to the vibrant hues in plants, especially fruits, flowers, and vegetables. Notably, they are recognized for imparting shades of blue, red, or purple to fruits, exemplified in berries and black currants.[42] The coloration of anthocyanins, glycosides derived from anthocyanidins, is subject to pH levels and

available substituting groups, with prevalent occurrences in the peels of diverse berries and currants. Beyond their aesthetic contribution, anthocyanins positively influence the cardiovascular system by inducing endothelium-dependent vasodilation. Moreover, they exhibit the capacity to lower BP in individuals with hypertension.[43]

2.6. Isoflavones

Abundant in leguminous plants, particularly soybeans, lentils, peas, and even certain microbes, isoflavones constitute a significant class of flavonoids.[44] Notably, soy isoflavones share structural similarities with mammalian estrogens, rendering them estrogen receptor agonists. Among the well-studied examples in this category are daidzein and genistein.[45] Enhanced consumption of isoflavones leads to the conversion of endogenous estrogens into their protective derivatives. Genistein, for instance, demonstrates an antihypertensive effect,[46] while daidzein plays a role in reducing oxidative stress-induced damage and low-density lipoprotein (LDL) oxidation, concurrently promoting the production of prostaglandins (PGIs) and NO.[47]

3. Flavonoids and vascular function

Numerous vasoactive compounds are released by endothelial cells, and these molecules are essential for controlling vascular shape and function in both healthy and pathological settings.[48] A broad class of compounds known as flavonoids interacts with a variety of targets and has a range of biological effects, suggesting that they may be used to treat a wide range of illnesses. Although the antioxidant effect was thought to be the main mechanism of action for flavonoids and polyphenols in the 1980s and 90s, new research has provided a deeper insight. It is now known that direct interactions with protein targets—particularly kinases—can modify signaling cascades. Moreover, a plausible reason for flavonoids' beneficial effects on human health is their pro-oxidant activity, which activates antioxidant defense systems such as the expression of antioxidant enzymes or the upregulation of endogenous antioxidant pathways.[49]

Despite their interactions with multiple targets, flavonoids are notably safe, possibly due to their longstanding presence in the mammalian diet, suggesting evolved mechanisms to mitigate toxicity.[50] NO is essential for preserving vascular homeostasis due to its integral roles in platelet aggregation, blood flow, vascular tone, and the regulation of VSMC proliferation. Endothelial dysfunction, which is characterized by decreased NO bioavailability, elevated oxidative stress, impaired endothelium-dependent vasodilation, and a prothrombotic, pro-inflammatory state of the vascular wall, is significantly influenced by disruptions in NO signaling pathways.[51]

A growing body of research relates diets high in flavonoids to improved vascular health, influencing endothelium-dependent vasorelaxation and NO bioavailability in both physiological and pathological settings. Nevertheless, little is known about the specific processes by which flavonoids enhance endothelium functions. Numerous molecular pathways, including endothelium- and NO-dependent relaxations, as well as antioxidant, anti-inflammatory, and antiproliferative qualities, have been postulated by research conducted in both human and animal models.[52,53]

In isolated arteries, flavonoids exhibit different vasodilator actions, varying in strength. Different mechanisms underlie these relaxing actions, particularly with regard to the function of NO and endothelium. Certain flavonoids, such as myricetin and epigallocatechin gallate (EGCG), increase the vasoconstrictor prostanoids generated from cyclooxygenase (COX), which might induce an endothelium-dependent contractile response.[54]

Certain flavonoids, such as quercetin and kaempferol, promote an endothelium-independent vasodilation mechanism in isolated arteries, contributing to a reduction in arterial pressure in experimental models.[55] Interestingly, compared to conductance vessels, the effects of these direct vasodilators are more noticeable in coronary and resistance arteries.[56] The suppression of intracellular Ca^{2+} release from the endoplasmic reticulum (ER), decrease in Ca^{2+} influx into VSMCs, and activation of ATP-sensitive potassium (KATP) channels are responsible for the endothelium-independent relaxant responses.[57]

Flavonoids contribute to improving endothelial dysfunction associated with cardiovascular diseases (CVDs) through increased endothelium- and NO-dependent relaxations. By heightening endothelium-derived relaxing factors, such as increased NO and H_2O_2 release, they induce acute endothelium-dependent vasodilation. Reactive oxygen species (ROS) released by endothelial cells drive this prooxidant process, which can be blocked by superoxide dismutase (SOD) and catalase (CAT).[58]

The availability of NO is the main mechanism underpinning the endothelium-dependent vasodilation caused by flavonoids. By upregulating the expression and activity of endothelial NO synthase (eNOS) and reducing NO degradation, flavonoids regulate the generation of NO.[59] Flavonoids like red wine polyphenols and delphinidin enhance eNOS mRNA expression in endothelial cells.[60] They modulate eNOS activity through a Ca^{2+}/calmodulin-dependent pathway, elevating intracellular Ca^{2+} levels, or through a Ca^{2+}-independent mechanism through phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (Akt)-dependent eNOS phosphorylation at the activation

site Ser1177 and dephosphorylation at the inhibition site Thr495.[61] The latter mechanism involves a prooxidant effect, as indicated by its prevention with permeant analogs of SOD.[20] However, the impact of isolated flavonols such as quercetin on eNOS expression and endothelial NO production in *in vitro* studies remains contentious, with conflicting results based on oxidative stress conditions.[62] Quercetin may scavenge NO under no oxidative stress, leading to its auto-oxidation and generation of O^{2-}, ultimately inactivating NO.[63] It could also reduce eNOS expression in tumor necrosis factor-alpha (TNF-α)-stimulated endothelial cells[64] and normalize upregulated eNOS in aortas from spontaneously hypertensive rats (SHR).[33] In aortic rings from normotensive and hypertensive animals, quercetin therapy over an extended period of time may not increase endothelium-dependent relaxation to insulin, indicating a direct inhibitory effect on PI3-K/Akt-dependent eNOS phosphorylation. However, due to its antioxidant qualities, quercetin may improve impaired endothelium-dependent vasodilation under conditions of increased oxidative stress, suggesting that NO production is not directly affected but rather that NO-dependent vasodilation is increased due to reduced O^{2}-driven NO inactivation.[33,65]

Moreover, flavonoids possess the ability to increase the production of NO in endothelial cells by suppressing the expression of caveolin-1 (Cav-1). Cav-1 functions as a significant negative regulator of eNOS activity in endothelial cells. This effect occurs through the activation of extracellular-signal-regulated kinases 1/2 (ERK1/2) and the inhibition of p38 mitogen-activated protein kinase (p38MAPK) signaling pathways.[66,67] In addition, flavonoids have been shown to inhibit various isoforms of phosphodiesterases, which play a critical role in NO-mediated relaxation and endothelium-dependent relaxation. It is worth noting that certain flavonoids, such as kaempferol, can enhance the relaxant response to the soluble guanylyl cyclase activator sodium nitroprusside.[69]

Flavonoid-mediated inhibition of endothelium-derived vasoconstrictors, particularly PGIs, has been proposed as a mechanism for preventing endothelial dysfunction, as evidenced by quercetin's action.[70] Conversely, flavonoids can indirectly induce vasodilation by enhancing NO bioavailability through several pathways, which include scavenging ROS, reducing endogenous eNOS inhibitors such as asymmetric dimethylarginine, blocking vasoconstrictor release (endothelin-1 [ET-1] and angiotensin II [Ang II]), and inhibiting enzymes involved in NO inactivation (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase, acetylcholinesterase, and angiotensin-converting enzyme [ACE]).[71]

Flavonoids' potent-free radical scavenging activity safeguards NO from ROS-mediated inactivation, preventing peroxynitrite and nitrotyrosine formation and contributing to their *in vivo* benefits.[72] In addition, they impede LDL oxidation, a key step in atherosclerotic plaque formation.[47] Their chelation of pro-oxidant metals further mitigates Fenton reactions and the generation of highly DNA-damaging hydroxyl radicals.[65] Beyond ROS scavenging, flavonoids enhance NO availability by inhibiting ROS-generating enzymes like 5-lipoxygenase, COX, xanthine oxidase, and NADPH oxidase, while upregulating antioxidant enzymes such as SOD, CAT, and peroxidase.[73] Moreover, they may prevent tetrahydrobiopterin oxidation and eNOS uncoupling, preserving eNOS function as an NO producer.[59] Recent evidence suggests that flavonoids can improve endothelial function under hypertensive and hyperglycemic conditions by attenuating ER stress through ROS reduction, likely due to their antioxidant properties. However, further research is needed to solidify the protective effects against ER stress-associated oxidative stress and vascular dysfunction.[36,56]

Flavonoids further contribute to endothelial protection by downregulating vasoconstrictor mediators, including Ang II, ET-1, and PGIs. Their interference with Ang II synthesis involves modulating ACE activity or its signaling pathways.[26,46] Quercetin and epicatechin, among others, suppress ET-1 production through Akt-mediated transcriptional regulation, demonstrated in both *in vitro* and *in vivo* studies.[27,55]

Molecular targets implicated in the vasculoprotective effects of flavonoids include arginase-2 (ARG2), nuclear factor erythroid 2-related factor 2 (Nrf2), and sirtuin 1 (SIRT-1). By reducing arginase-2 activity, flavonoids prevent L-arginine competition with eNOS, subsequently inhibiting NO formation.[74] Activation of Nrf2 by quercetin and EGCG upregulates antioxidant enzymes via the Nrf2/ARE pathway, enhancing cellular defense against oxidative stress.[75] SIRT-1 directly interacts with eNOS, deacetylating it at Lys496 and Lys506 to promote vasodilation and enhance eNOS activity.[76] Given that downregulation or pharmacological inhibition of SIRT-1 is closely linked to endothelial dysfunction,[77] recent evidence suggests that quercetin may act as a SIRT-1 activator, potentially ameliorating impaired endothelial function.[78]

The vasculature harbors inherent anti-inflammatory and anti-proliferative properties that augment the potential antihypertensive and antiatherosclerotic effects of flavonoids. In the context of vascular pathology, inflammation stands as a pivotal element, characterized by the excessive production of proinflammatory agents and adhesion molecules, frequently orchestrated by nuclear

factor kappa-light-chain-enhancer of activated B cells (NF-κB). Activation of NF-κB leads to the upregulation of pro-inflammatory mediators such as inducible NO synthase (iNOS), COX-2, TNF-α, and interleukin-6 (IL-6). Moreover, NF-κB activation is intricately associated with the mitogen-activated protein kinases (MAPKs), pivotal components in the propagation of pro-inflammatory responses correlated with endothelial dysfunction. A plethora of investigations substantiate the inhibitory effects of flavonoids on the redox-sensitive NF-κB/MAPK signaling pathway, thereby demonstrating their anti-inflammatory properties.[79-81]

Flavonoids exhibit the capacity to impede the proliferation, migration, and tube formation of endothelial cells, potentially facilitating their antiangiogenic properties. This effect often correlates with a reduction in the expression of vascular endothelial growth factor.[83,84] Additionally, flavonoids have demonstrated the ability to inhibit proliferation, mitigate hypertrophy, or induce apoptosis in VSMCs in culture, thereby potentially limiting vascular remodeling. These inhibitory effects on VSMC hypertrophy and DNA synthesis are believed to be linked to reduced MAPK activity.[85,86]

Despite the potential of flavonoid-rich foods to increase NO bioavailability and combat vascular disorders, their low *in vivo* bioavailability remains a significant hurdle in clinical applications. This limitation is attributed to poor absorption, rapid metabolism, and degradation. Clinical studies have reported significantly lower plasma concentrations of orally administered flavonoids compared to those used in *in vitro* and *in vivo* experiments, potentially insufficient to achieve protective effects on endothelial function.[63,87] Overcoming this challenge hinges on addressing the factors contributing to low bioavailability, including extensive metabolism and the impact of gut microbiota on absorption.[88-90]

4. Flavonoids-induced effects in relation to vasodilation

4.1. Antiplatelet effects

Platelets, smaller blood corpuscles, are pivotal in the coagulation process for hemostasis, preventing bleeding.[66] Intact blood vessels inhibit clotting, while any injury activates them. The regulation of this process occurs through the endothelial lining, which secretes inhibitors of coagulation and platelet aggregation when undamaged. However, on rupture, this endothelial lining releases von Willebrand factor (vWF), a crucial molecule maintaining hemostasis in humans.[67] On injury to blood vessels, local sympathetic pain receptors trigger an immediate reflex response. The damaged vessels undergo constriction to minimize the size of the injury and reduce blood loss, resulting in vascular spasms. Collagen, found in the vessel walls, becomes exposed to the blood when vessels are damaged, promoting platelet adhesion at the injury site where collagen fibers are exposed. On contact with this collagen, platelets release cytoplasmic granules containing serotonin, ADP, and thromboxane A_2 (TXA_2), leading to vasoconstriction.[66] To address the remaining issue, platelets activate each other and simultaneously initiate the coagulation cascade to form a blood clot. Platelet activation plays a central role in both protective hemostasis and pathological thrombosis through various physiological pathways. Excessive platelet aggregation is closely linked to several chronic diseases, including diabetes, hypertension, and various CVDs.[68]

High concentrations of adhesion proteins, resulting from excessive platelet activation, lead to the generation of thrombi, contributing to the development of various thrombotic diseases.[69] This development is primarily due to thrombi clogging narrow blood vessels, causing blockages in or near the affected areas. TXA_2, released during platelet activation, acts as a potent vasoconstrictor and platelet activator. Synthesized in platelets by the cytoplasmic COX enzyme from arachidonic acid sourced from platelet membranes, it undergoes conversion into TXA_2 through either the TXA or PGI pathways. This conversion process, facilitated by COX-1, is crucial for platelet activation.[70] Given TXA_2's role in platelet recruitment and activation, blocking COX can potentially reduce platelet activation and aggregation at or near the injury site. Flavonoids demonstrate several beneficial effects, including improved endothelial functioning, reduced platelet adhesion, and interference in lipid metabolism.[71]

Flavonoids function as antagonists of TXA_2 receptors located on the platelet membrane, influencing TXA2 receptor levels that regulate COX expression. The presence of flavonoids indirectly inhibits COX-1 activity, resulting in platelet inactivation. Structural features of flavonoids, such as double bonds at C2 – C3 and a keto group at C4, enable tight stearic binding to TXA_2 receptors. Non-glucuronidated flavonoids such as genistein and daidzein hinder platelet aggregation by binding to TXA_2 surface receptors.[72] Collagen, a key initiator of platelet aggregation, is influenced by flavonoids, which not only intervene in arachidonic acid metabolism but also impact collagen metabolism, reducing platelet aggregation. Flavonoids minimize oxidative stress, a trigger for collagen-induced platelet aggregation, by acting as NADPH oxidase inhibitors.[73] Plant extracts containing flavonoids exert antiplatelet effects through various mechanisms, including the inhibition of intracellular Ca^{2+} to prevent

thrombi formation and cytoskeletal reorganization. Other mechanisms involve inhibiting pathways like thrombocyte secretion, protein C breakdown, platelet activation factor, and phospholipase C. Antiplatelet activity is also achieved by blocking TXA formation and increasing intracellular cAMP and cGMP levels. Flavonoids inhibit enzymes like phospholipase A and certain tyrosine kinases.[74]

Glycoprotein (GP) Ib/IIa inhibitors, another facet of flavonoid action, bind to platelet receptors, preventing fibrinogen and vWF binding crucial for platelet aggregation. Flavonoids like quercetin or catechin elevate NO levels, decreasing the expression of the GP Ib/IIa complex and thereby blocking platelet aggregation *in vitro*.[75]

4.2. Hypertension

Hypertension is a clinical condition characterized by persistently elevated diastolic and/or systolic BP within the blood vessels.[76] It is a leading cause of illness and mortality. Contributing factors include obesity, a sedentary lifestyle, smoking, stress, and aging.[77] NO, synthesized by the endothelium, plays a pivotal role in vascular health. Functioning as a vasodilator, NO regulates vascular tone by inducing relaxation in blood vessels, thereby influencing BP (Table 1). On release into the bloodstream by the endothelium, NO either binds to hemoglobin or diffuses into smooth muscles.[78]

Within smooth muscles, NO binds and activates guanylyl cyclase, initiating the cGMP-protein kinase G cascade pathway. This cascade involves a series of chemical reactions within the effector cell, culminating in smooth muscle relaxation. Activation of the cGMP-protein kinase G cascade leads to several mechanisms that enhance smooth muscle relaxation through elevated levels of cGMP, including the inhibition of Ca^{2+} uptake within cells, activation of K^+ channels resulting in hyperpolarization, and subsequent vasodilation.[86] Another mechanism involves the phosphorylation of myosin light chains by activated cGMP-dependent kinase, further reducing vasoconstriction in vascular smooth muscles.[87]

Vasodilation, achieved through these mechanisms, widens the blood vessel lumen, facilitating increased blood flow and reducing hypertension. Flavones exhibit antihypertensive activity by targeting the cGMP protein kinase G cascade. They activate the cGMP protein kinase A cascade, leading to excessive synthesis and secretion of endothelial NO. This, in turn, induces vasodilation by modulating Ca^{2+} and K^+ ion levels, promoting hyperpolarization of smooth muscles adjacent to the endothelium.[55]

Flavonols, such as kaempferol and quercetin, demonstrate their antihypertensive effects through the modulation of the RAAS. This regulatory system plays a crucial role in BP control by influencing factors such as blood volume, electrolyte balance, and vasodilation. Kaempferol and quercetin interact with various components of the RAAS to exert their antihypertensive actions.[88] The renin-mediated conversion of angiotensinogen to Ang I and the subsequent ACE-mediated conversion of Ang I to Ang II constitute the RAAS pathway. Ang II, a potent vasoconstrictor, also stimulates aldosterone secretion, leading to sodium and water retention.[89] Studies indicate that kaempferol and quercetin can inhibit ACE activity, thereby reducing Ang II production and preventing excessive vasoconstriction, promoting vasodilation, and contributing to an overall reduction in BP. In addition, these flavonols may influence aldosterone production, assisting in maintaining electrolyte balance and preventing fluid retention. The ability of kaempferol and quercetin to modulate key components of the RAAS underscores their potential as natural compounds for hypertension management.[55] By targeting crucial elements of this regulatory system, these flavonols provide a holistic approach to BP control, addressing both vascular tone and fluid balance. It is important to note that the specific mechanisms through which flavonols interact with the RAAS may vary, and ongoing research aims to explore these pathways in detail.

An important independent risk factor for cardiovascular events, such as coronary heart disease and stroke, is high BP. Many different pathways influence hypertension, a complex illness that is frequently necessary but has no clear etiology.[90] Despite the availability of numerous antihypertensive drugs, many patients still struggle with suboptimal BP control, posing a heightened risk of cardiovascular complications. Consequently, efforts to reduce hypertension prevalence have focused on non-pharmacologic approaches, with dietary measures emerging as effective strategies.[91] Increasing fruit and vegetable intake, aligned with guidelines for modulating hypertension, is particularly noteworthy. Apart from popular dietary strategies such as the Mediterranean diet and the dietary approaches to stop hypertension diet, flavonoids and dietary sources containing flavonoids have garnered interest due to their potential to decrease BP.[92]

Evidence from observational studies, clinical trials, and meta-analyses supports the positive impact of flavonoid-rich foods on hypertension. Diets rich in vegetables and fruits, fruit juices, berries, green, and black tea, as well as cocoa and dark chocolate, have been associated with reduced BP.[93] Notably, cocoa may exhibit superiority among flavonoid sources in effectively lowering BP.[94]

Research suggests that increased NO bioavailability, in conjunction with antioxidant and anti-inflammatory

Table 1. Studies ınvestigating the effects of quercetin, a flavonoid variety, on blood pressure regulation

Study	Details and findings of the study
Dehghani *et al.*[79]	(i) The study involved 88 post-MI patients who were randomly assigned to receive either 500 mg/day of quercetin or a placebo for eight weeks in a double-blind, placebo-controlled trial. The researchers aimed to assess the effects of quercetin supplementation on inflammatory factors, total antioxidant capacity (TAC), and quality of life in these patients. (ii) Results indicated that quercetin supplementation significantly increased serum TAC levels compared to the placebo group. Levels of TNF-α, an inflammatory marker, decreased in the quercetin group.
Dower *et al.*[80]	(i) In this study, researchers investigated the effects of supplementation with pure epicatechin and quercetin on vascular function and cardiometabolic health in apparently healthy individuals with moderately elevated blood pressure. (ii) Study participants, aged 40 – 80 years, were enrolled in a randomized, double-blind, placebo-controlled crossover trial. They received either (-)-epicatechin (100 mg/d), quercetin-3-glucoside (160 mg/d), or placebo capsules for four weeks in random order. The primary outcome measured was the change in flow-mediated dilation, a marker of vascular function, from pre- to post-intervention. (iii) Results indicated that epicatechin supplementation did not significantly change flow-mediated dilation but improved fasting plasma insulin levels and insulin resistance.
Edwards *et al.*[81]	(i) In this study, researchers investigated the potential of quercetin supplementation to lower blood pressure in hypertensive patients based on prior epidemiological studies linking quercetin intake to reduced risk of coronary heart disease and stroke. (ii) The study enrolled men and women with prehypertension and stage 1 hypertension in a randomized, double-blind, placebo-controlled crossover trial. Participants received either 730 mg of quercetin daily or a placebo for 28 days. (iii) Results showed that quercetin supplementation did not significantly alter blood pressure in prehypertensive patients. However, in patients with Stage 1 hypertension, quercetin treatment led to reductions in systolic, diastolic, and mean arterial pressures. Notably, these reductions were observed without significant changes in systemic markers of oxidative stress, contrary to findings in animal studies.
Nishihira *et al.*[82]	(i) In this randomized, double-blind, placebo-controlled clinical trial, researchers investigated the potential cognitive benefits of quercetin-rich onion intake in healthy Japanese individuals aged 60 – 79 years old. (ii) Seventy participants were divided into two groups: one receiving quercetin-rich onion (the active test food) and the other receiving quercetin-free onion as a placebo for 24 weeks. The cognitive function of participants was assessed using various tests, including the Mini-Mental State Examination, Cognitive Assessment for Dementia iPad version, and Neuropsychiatric Inventory Nursing Home version. (iii) Results showed that the group consuming quercetin-rich onion experienced significant improvements in Mini-Mental State Examination scores compared to the placebo group after 24 weeks. In addition, the active test food group showed improvements in emotional function evaluation, suggesting a reduction in depressive symptoms and increased motivation.
Shatylo *et al.*[83]	(i) In this randomized, placebo-controlled, double-blind clinical trial, researchers investigated the effects of quercetin supplementation on various aspects of metabolic syndrome (MetS) in patients aged 60 and above. (ii) Participants consumed two quercetin-containing or placebo tablets three times per day for a duration of 3 months, resulting in a daily quercetin dose of 240 mg. (iii) Results showed that quercetin administration led to significant improvements in several parameters of MetS. These included reductions in body weight and body mass index, as well as decreases in systolic and diastolic blood pressure. Quercetin intervention also improved cholesterol metabolism, as evidenced by reductions in serum total cholesterol and low-density lipoprotein cholesterol levels. In addition, fasting plasma insulin and glucose levels at the 2-h oral glucose tolerance test were reduced following quercetin supplementation.
Shi and Williamson[84]	(i) In this randomized, double-blinded, placebo-controlled, cross-over trial, researchers investigated the effects of oral supplementation of quercetin on plasma uric acid, blood pressure, and fasting glucose levels. (ii) Twenty-two healthy males aged 19 – 60 years with baseline plasma uric acid concentrations in the higher, yet still considered healthy, range was recruited for the study. (iii) Participants received one tablet containing 500 mg of quercetin daily for 4 weeks, compared to placebo, with a 4-week washout period between treatments. The primary outcome measured was the change in plasma uric acid concentrations after 2 and 4 weeks, while secondary outcome measures included changes in fasting plasma glucose, 24-h urinary excretion of uric acid, and resting blood pressure. (iv) Results showed that after quercetin treatment, plasma uric acid concentrations were significantly lowered by−26.5 μmol/L compared to placebo (95% CI, −7.6 – −45.5; P=0.008). (v) The study concluded that daily supplementation of 500 mg of quercetin for 4 weeks, equivalent to the bioavailable amount found in approximately 100 g of red onions, significantly reduces elevated plasma uric acid concentrations in healthy males.

(Cont'd...)

Table 1. ***(Continued)***

Study	Details and findings of the study
Ali *et al.*[85]	(i) This study investigated the antihypertensive properties of quercetin and its underlying mechanisms in angiotensin II (Ang II)-induced hypertension. (ii) In Ang II-infused C57BL/6 mice, quercetin treatment significantly reduced the increase in blood pressure, pulse wave velocity, and aortic thickness of the abdominal aorta. RNA sequencing revealed that quercetin treatment reversed 464 differentially expressed transcripts in the abdominal aorta of Ang II-infused mice. (iii) Further analysis identified common pathways affected by quercetin treatment, including cell cycle and p53 pathways, which play crucial roles in regulating vascular function and proliferation. Immunohistochemistry confirmed that quercetin decreased the expression of proliferating cell nuclear antigen, cyclin-dependent kinase-4 (CDK4), and cyclin D1 while increasing the expression of p53 and p21 in abdominal aortic tissues. (iv) *In vitro* studies with vascular smooth muscle cells (VSMCs) showed that quercetin treatment decreased cell viability, arrested cell cycle progression at the G0/G1 phase, and upregulated the expression of p53 and p21 proteins. In addition, quercetin downregulated the expression of cell cycle-related markers CDK4 and cyclin D1 in Ang II-stimulated VSMCs.

Abbreviation: TNF-α: Tumor necrosis factor-alpha.

qualities, may be the primary ways that diets high in flavonoids reduce hypertension.[55] Notably, similar antihypertensive outcomes have been observed in pre-clinical and clinical studies when products enriched with flavonoids or flavonoid extracts were administered.[30,93,95] However, contrasting results have emerged, especially in trials related to red wine, tea, soy, and chocolate. The differences in study designs, participant heterogeneity, dosage differences, varying flavonoid concentration and bioavailability, and the inclusion of alcohol, high-calorie food (sugar and saturated fat), and caffeine in certain products can all contribute to the variance. These variations highlight the need to investigate individual pure flavonoids.

The capacity of the main dietary flavonoids from five regularly ingested subgroups—flavonols and anthocyanins—to lessen or ameliorate an increase in BP is outlined in a thorough review by Clark *et al.*[96] All of these flavonoids have shown antihypertensive benefits in a variety of animal models and clinical trials; however, we shall concentrate on quercetin and epicatechin due to their exceptional capacities in consistently lowering BP.

Particularly, quercetin has demonstrated antihypertensive benefits in widely used preclinical models of hypertension. Initial reports on its effects were conducted on SHR, a model mirroring human hypertension.[30] Further investigations have confirmed and extended these results in various hypertensive rodent models, such as Goldblatt rats with two kidneys and one clip, rats with constricted aortas, rats treated with Nω-nitroL-arginine methyl ester (L-NAME), rats infused with Ang II, rats with hypertension induced from deoxycorticosterone acetate (DOCA)-salt, rats with hypertension induced from sensitivity to Dahl salt, and rats with hypertension induced from sodium chloride (NaCl).[97] The effectiveness of quercetin in lowering the increased BP has also been shown in metabolic syndrome models, which include Zucker rats that are obese or animals who are fed a fat- and sugar-rich diet. The range of chronic dosages often utilized in these studies is 2 – 300 mg/kg/day, with the most regularly used dose being 10 mg/kg/day.[98]

Carresi *et al.*[99] examined the effects of a polyphenol-rich fraction of bergamot (BPF) on renovascular hypertension (RVH) and associated reno-cardiac diseases using a rat model. They induced hypertension in adult male Wistar rats through unilateral renal artery ligation and treatment with DOCA and 1% NaCl water. The rats were divided into groups receiving DOCA and NaCl water treated with BPF gavage treatment or subcutaneous injection of vehicle treatment (control). Results revealed that rats subjected to renal artery ligation and DOCA treatment experienced increased mean arterial BP, resistive index of contralateral renal artery flow, and kidney volume, along with dysfunction in cardiac tissue strain and dyssynchrony in cardiac wall motion. These rats also exhibited elevated levels of pro-inflammatory cytokines and chemokines and increased expression of neutrophil gelatinase-associated lipocalin (NGAL) in the ligated kidney. Treatment with BPF prevented the increase in BP, protected the contralateral kidney volume, and ameliorated cardiac tissue strain dysfunction and dyssynchrony. Furthermore, BPF reduced circulating levels of pro-inflammatory cytokines and chemokines and restored NGAL levels in the kidney.

In human epidemiological research, a negative correlation has been observed between dietary quercetin intake and hypertension. Several clinical investigations have demonstrated a drop in BP following the consumption of pure quercetin. For instance, Edwards *et al.*[81] demonstrated BP-lowering effects without systemic markers of oxidative stress reduction in patients with stage 1 hypertension. In addition, Egert *et al.*[100] revealed that quercetin lowered BP in overweight individuals with a high CVD risk profile and in overweight-obese

carriers of the apo epsilon3/epsilon3 genotype, but not in epsilon4 allele carriers. Lee *et al.*[101] investigated the effect of quercetin on cardiometabolic risks in healthy male smokers and observed notable drops in both systolic and diastolic BP in the quercetin-rich supplementation group. Similarly, Larson *et al.*[102] reported a decrease in BP in men with Stage 1 hypertension using acute quercetin aglycone in a double-blind, placebo-controlled crossover design. However, this reduction did not coincide with changes in ACE activity, ET1 levels, or NO bioavailability, and there were no alterations in vascular reactivity. These findings are noteworthy as these mechanisms are typically considered principal contributors to BP reduction. Furthermore, quercetin intake exhibited a capacity to reduce systolic BP in women with Type 2 diabetes.[103] However, inconsistent results have been observed, with some studies reporting no significant BP reduction in hypertensive subjects.[103,104]

A recent meta-analysis combining the results of seven randomized, placebo-controlled clinical trials demonstrated a substantial drop in BP with quercetin supplementation. This effect may have been stronger at dosages over 500 mg/day, which are higher than those typically used. In fact, Vogiatzoglou *et al.*[105] showed that the typical European intake of flavonoids is lower than the dosages linked to notable health impacts. Pérez *et al.*[106] discovered that quercetin had a vasodilator effect on the arteries of young, healthy humans; however, this increase in diameter in a major conduit artery did not result in changes in BP. This finding is crucial for the prevention of hypertension in normotensive individuals, as no reduction in BP was observed in normal controls in pre-clinical and clinical studies.[55] Therefore, it appears that a certain degree of elevated BP may be a prerequisite for quercetin to exert its BP-lowering effect.[30]

The positive effects of quercetin on BP have been attributed to various mechanisms. These mechanisms include ameliorating endothelial dysfunction through the activation of eNOS, increasing NO bioavailability, direct vasodilatory action, and exhibiting antioxidant and anti-inflammatory properties.[55,57,102] In addition, quercetin's BP-lowering effect may involve direct renal protection.[32] However, it remains unclear whether the *in vivo* antihypertensive effects are attributed to quercetin itself or its metabolites.[107] Despite these insights, the exact mechanisms responsible for quercetin's BP-lowering effect remain partially unknown, with some studies presenting conflicting results.[107]

Apples, grapes, tea, and chocolate contain significant levels of epicatechin, which is a well-known bioactive flavanol. In experimental hypertension rat models, a number of polyphenolic extracts with a high flavanol content, including cocoa extract, black or green tea, and red wine polyphenols, have demonstrated the ability to lower BP.[14,94]

Studies on animals indicate that effective doses of pure epicatechin for antihypertensive effects range from 10 to 350 mg/kg/day. It is noteworthy that doses below 5 mg/kg/day showed no signs of lowering BP. In adult DOCA-salt rats, Gómez-Guzmán *et al.*[108] found that epicatechin significantly lowered BP and enhanced endothelium-dependent vasorelaxation. Other hypertension models, including fructose-induced hypertension, L-NAME-induced hypertension, and SHR, have also shown the BP-lowering benefits of epicatechin.[109]

Various studies have delved into the mechanisms underlying epicatechin's antihypertensive effects. In L-NAME-induced hypertension models, epicatechin prevented BP increases, reduced oxidative stress, and restored NO bioavailability.[109,110] However, Gómez-Guzmán *et al.*[108] highlighted that the antihypertensive effects of epicatechin are dependent on the duration, dose, and specific disease conditions, showing antioxidative and anti-inflammatory benefits but no significant effect on the development of hypertension with chronic epicatechin administration. Additional effects of epicatechin include lowering plasma levels of COX-2 and ET-1, inhibiting ACE activity, improving the redox condition of cardiac tissue, improving vascular NO bioavailability to improve endothelial function, and modulating cell signaling pathways.[111] These multifaceted effects contribute to epicatechin's antihypertensive properties.

There is a dearth of research on pure epicatechin in human trials, despite strong data supporting the cardiovascular benefits of products enriched with or containing epicatechin in human investigations. While some clinical research has examined its potential benefits, more studies are necessary to draw firm conclusions regarding epicatechin's potential as an antihypertensive agent.[112,113]

Dietary flavonoids, a diverse group of polyphenolic compounds, exhibit varied mechanisms for mediating BP-lowering effects. Unlike quercetin, which has no effect on ACE activity, epicatechin appears to depend on the suppression of ACE activity for its antihypertensive effects.[114] The structural diversity of flavonoid subgroups makes it challenging to pinpoint a singular mechanism. Overall, flavonoids are proposed to interact chemically with ROS and induce changes in various enzymes, ion channels, and transcription factors.[18] Potential mechanisms contributing to the BP-lowering effects of flavonoids include the improvement of endothelial function,

reduction in ROS, and direct renal effects.[71] However, these mechanisms remain subjects of ongoing research.

In summary, research on humans and animals has shown that a number of flavonoids, most notably epicatechin and quercetin, have antihypertensive effects. However, it is noteworthy that these benefits are primarily observed when BP is elevated. Therefore, flavonoids could be viewed as an adjunct to antihypertensive medication. Nevertheless, more investigation is needed to determine the precise pathways underlying the BP-lowering effects and the therapeutic significance of each flavonoid molecule.

5. Flavonoids and atherosclerosis

Under specific conditions, the walls of blood vessels may undergo a process of filling and thickening due to the accumulation of substances carried by the blood or produced within the vessel itself. These substances include cholesterol, fatty acids, calcium, and fibrin, as well as various cells such as red blood cells, platelets, smooth muscle cells, fibroblasts, and macrophages. This process, termed arteriosclerosis, results in the hardening, stiffening, and narrowing of the vessel at the affected site, thereby reducing the blood supply to the surrounding tissue. Factors such as obesity, an unhealthy diet, or lipid metabolism issues can contribute to the accumulation of cholesterol, fatty substances (triglycerides and LDL), fibrin, calcium, and extracellular matrix components within the vessel wall. In response to these accumulations, macrophages infiltrate the vessel walls and phagocytize oxidized LDL (ox-LDL) due to heightened levels of oxidative stress, transforming into "foam cells" due to the presence of fat droplets within them. This infiltration gives rise to the characteristic fatty streaks observed in atherosclerotic vessels. The amalgamation of substances from the blood and vessels, combined with infiltrated vascular cells, forms a structure known as a "plaque," which significantly alters the vessel wall, making it more rigid and narrower. This alteration results in a specific type of arteriosclerosis known as atherosclerosis.[115]

Over time, atherosclerotic plaques can grow to the extent of creating a thrombus capable of obstructing the vessel and causing tissue ischemia. Alternatively, the plaque may become unstable, rupture, and release fragments, leading to emboli that could cause an embolism by blocking a vessel anywhere in the body. Consequently, atherosclerosis is a chronic disease that develops silently over many years until sudden clinical manifestations appear, including ischemic heart disease, stroke, and peripheral arterial disease. Established risk factors implicated in the pathogenesis of atherosclerosis include chronic inflammation, oxidative stress, elevated levels of blood cholesterol and lipids, obesity, smoking, advancing age, hypertension, familial predisposition, endothelial dysfunction, suboptimal dietary patterns, insulin resistance, and diabetes.[116]

While mortality rates attributable to ischemic heart disease or stroke have markedly diminished in developed nations since 1990s, ischemic heart disease persists as the foremost cause of premature mortality among adults. Consequently, atherosclerosis stands out as the most prevalent and, consequently, the most hazardous form of arteriosclerosis.[117] As mentioned earlier, the onset and development of atherosclerosis involve multiple processes.

Flavonoids exhibit a wide range of mechanisms of action, including the inhibition and activation of enzymes, the control of gene and protein expression, and functions such as cytotoxic, anti-ulcerogenic, anti-microbial, antiviral, anti-neoplastic, hepatoprotective, antihypertensive, lipid-lowering, antiplatelet, and anti-inflammatory effects.[118] Due to these diverse actions, flavonoids can influence the course of the disease, preventing or reducing its severity, whether *in vitro*, *in vivo*, or as observed in clinical trials.

Historically, doubts existed about the benefits of flavonoid intake in preventing atherosclerosis due to contradictory results, variations in doses used in studies, and a lack of *in vivo* data.[119] While some challenges persist, such as the non-standardization of compounds and doses, and the need for more clinical studies with single flavonoids, accumulated results have rendered these concerns outdated. Clear evidence now exists that several flavonoids can prevent and mitigate atherosclerosis, demonstrated in both *in vitro* cultures and *in vivo* animal models. As an illustration, quercetin, one of the extensively investigated flavonoids, has demonstrated efficacy in mitigating the advancement of atherosclerosis. In a recent investigation utilizing apolipoprotein E deficient ($ApoE^{-/-}$) mice, a common animal model for atherosclerosis research, the administration of a diet enriched with 0.1% (w/w) quercetin over a duration of 20 weeks effectively suppressed dendritic cell activation and the inflammatory response, notably impeding the progression of atherosclerotic pathology observed in untreated counterparts. Moreover, this intervention yielded a notable 30% decrease in atherosclerotic lesions within the aortae.[120] In a subsequent *in vitro* study, the observed effects were attributed to quercetin's ability to downregulate dendritic cell activation by increasing disabled 2 (Dab2) protein expression, thereby inhibiting the pivotal NF-κB inflammatory pathway. Furthermore, the O-methylated flavone nobiletin exhibited its capacity to inhibit the NF-κB inflammatory pathway in human umbilical endothelial cells (HUVECs), indicating potential antiatherogenic effects.[121] Earlier research had already indicated that quercetin surpassed

other flavonoids in efficacy for slowing atherosclerosis progression and reducing plaque size. These findings were associated with enhanced endothelial function, characterized by increased vascular endothelial eNOS activity, and modulation of oxidative stress status, evidenced by elevated heme oxygenase-1 protein levels and nitrate excretion. In addition, a decrease in inflammatory markers was noted, including reduced levels of vascular superoxide anion (O_2^-) and leukotriene B4, as well as decreased aortic F2-isoprostane and plasma P-selectin concentrations. Moreover, in the same investigation, the dimeric catechin theaflavin demonstrated the potential to diminish the size of atherosclerotic lesions and improve some of these parameters, albeit with lesser potency.[121]

However, these results are not the sole evidence supporting the *in vivo* antiatherogenic properties of quercetin. In a similar ApoE$^{-/-}$ mouse model, this time fed a high-fat diet for 24 weeks, quercetin supplementation reduced the atherosclerotic plaque area in the aorta, particularly by 27.7% with the high dose of quercetin, closely aligning with the 30% reduction observed in the aforementioned study. Furthermore, this intervention resulted in a dose-dependent decrease in macrophage infiltration within the atherosclerotic lesion, reduced accumulation of ox-LDL, diminished systemic oxidative stress, and suppressed expression of NADPH oxidase subunits p47phox and p67phox in the aorta compared to untreated mice. In addition, *in vitro* experiments demonstrated that quercetin could impede the translocation of p47phox to the cell membrane and mitigate NADPH oxidase activation induced by ox-LDL in mouse peritoneal macrophages.[122] Remarkably, alongside quercetin's systemic antiatherogenic characteristics, its glucuronides, notably quercetin-3-glucuronide (Q3GA), the principal quercetin metabolite detected in human blood, selectively accumulate within atherosclerotic lesions in the human aorta, predominantly within foam cells originating from macrophages. Even subsequent to deconjugation and conversion back to quercetin aglycone, these metabolites maintain their activity.[123]

According to a recent meta-analysis,[120] of the 18 flavonoids examined, quercetin and its metabolites were the most effective in reducing aortic atherosclerotic lesions in the ApoE$^{-/-}$ mouse model. While not as well researched as quercetin, other flavonoids also show significant antiatherosclerotic qualities, primarily by blocking other pro-inflammatory mediators and pathways. For example, dihydromyricetin has demonstrated antiatherogenic benefits in the atherosclerotic model using LDL receptor-deficient (LDLr$^{-/-}$) mice fed a high-fat diet. Like quercetin, it decreases oxidative stress, ox-LDL generation, and inflammatory indicators, enhances endothelial function, and lowers high serum lipid levels. It also inhibits the formation of macrophage-derived foam cells.[124]

When dyslipidemia was examined for its effects on cerebral artery flow and structure, catechin was shown to shield atherosclerotic mice from alterations in the compliance and structure of the arterial wall. This protective strategy was associated with improved endothelial function, normalization of pro-metalloproteinase-9 (MMP-9) activity, and a decrease in ROS produced as a result of oxidative stress.[125] Different flavonoids show similar effects, albeit with varying levels of intensity, suggesting different levels of efficacy against atherosclerosis. Supporting this idea, male C57BL/6 mice that received a 0.6% w/w luteolin supplement for 3 weeks showed significant reductions in TNF-α-induced vascular inflammation. This supplementation reduced serum levels of chemokines, such as IL-8, ICAM-1, and the mouse homolog of human monocyte chemotactic protein-1 (MCP-1). Luteolin decreased VCAM-1 and monocyte-derived macrophages in the aorta in comparison to untreated animals, due to its ability to impede monocyte adherence to the endothelium, a critical stage in the subsequent development of foam cells in atherosclerotic plaques. Further *in vitro* tests revealed that luteolin exerted comparable effects by inhibiting the NF-κB pathway and thus decreasing TNF-α-stimulated production of MCP-1 and adhesion molecules ICAM-1 and VCAM-1.[126]

In high-fat-fed mice, naringenin, a flavonoid, showed promise in suppressing macrophage infiltration into adipose tissue during a brief 14-day treatment at a dose of 100 mg/kg/day. This effect was linked to MCP-1 inhibition, similar to luteolin. Compared to untreated high-fat-fed mice, naringenin-treated high-fat-fed mice showed improvements in body weight, blood glucose, or lipid profile.[127] Another flavonoid, baicalin, showed inhibitory effects on MCP-1, VCAM-1, and IL-6 in the kidneys of high-cholesterol-fed ApoE$^{-/-}$ mice when administered at a dose of 100 mg/kg/day for 12 weeks, thereby maintaining renal function.[128]

Flavonoids are instrumental in shielding the endothelium from oxidative damage caused by ox-LDL. In a study, pretreatment with EGCG shielded HUVECs from oxidative harm induced by ox-LDL. This protection was evidenced by heightened expression of eNOS, improved endothelial function, prevention of iNOS induction, and reduced expression of NADPH oxidase subunits. Moreover, EGCG pretreatment reversed the ox-LDL-induced alterations in the Jagged-1/Notch pathway, indicating its pivotal role in the observed protective effects.[129]

Flavonoids aid in the removal of cholesterol from macrophages and also play a part in inhibiting the

oxidation of LDL and the production of foam cells. In a study conducted on RAW264.7 macrophages, researchers observed a significant impact of chrysin treatment on cellular cholesterol levels and its transport mediated by high-density lipoprotein (HDL). RAW264.7 macrophages are a commonly used cell line for studying macrophage biology and lipid metabolism. On exposure to varying concentrations of chrysin, the researchers noted a dose-dependent increase in the export of cholesterol mediated by HDL. Furthermore, the study revealed a concurrent decrease in intracellular cholesterol levels following chrysin treatment. This reduction in cellular cholesterol content indicates that chrysin effectively decreases the amount of cholesterol stored within macrophages. Importantly, the magnitude of this reduction was comparable to that achieved with lovastatin, a well-known hypocholesterolemic drug commonly prescribed for lowering cholesterol levels in patients at risk of CVD.[130]

One promising therapeutic avenue for the prevention and treatment of atherosclerosis is the impact of flavonoids on reverse cholesterol transport.[131] A recently elucidated mechanism through which certain flavonoids operate involves the augmentation of paraoxonase (PON) proteins' activity or expression. PONs are enzymes associated with anti-inflammatory, antioxidative, and antiatherogenic properties.[132] Research suggests that in cultured mouse macrophages, quercetin and isorhamnetin can upregulate PON2 gene expression. Nevertheless, quercetin failed to elicit the same effects in humans, most likely due to conjugation with glucuronic acid, which reduced PON2-inducing activity, demonstrating the variability of the actions of flavonoids and their metabolites.[133] Examining quercetin's impact on PON1 gene expression and activity revealed similar results.[134] In addition, by raising PON1 activity, shielding LDL from oxidation, and reducing lipid peroxidation, quercetin showed anti-atherosclerotic benefits in a rat model exposed to oxidative damage induced by mercuric chloride.[135] While not as potent, catechin exhibited similar effects. Recent research has further investigated the effect of flavonoids on PON activity in humans using extracts and enriched meals, confirming the compounds' ability to increase PON expression or activity.[20]

MicroRNAs represent a burgeoning field of research for CVD. These small non-coding RNAs regulate gene expression and protein expression, playing a pivotal role in various vascular processes and being altered in pathological states such as hypertension, atherosclerosis, and diabetes.[136] For example, in dyslipidemic obese rats, long-term proanthocyanidin treatment reversed elevated levels of miR-33a, miR-21, miR-122, miR-3064-5p, and miR-122, which are important regulators of liver lipid metabolism.[137] Furthermore, in RAW264.7 macrophages challenged with lipopolysaccharide, quercetin, and isorhamnetin downregulated the proinflammatory miR-155, although the metabolite Q3GA did not show this impact.[138]

Two recently identified anti-atherosclerotic actions of flavonoids — the modulation of gut microbiota activity and miRNA activity — have been connected in a significant study. Similar to chrysin, protocatechuic acid, a metabolite of the anthocyanin cyanidin-3-O-β-glucoside generated by gut microbiota action, has shown antiatherogenic properties in $ApoE^{-/-}$ mice by reversing cholesterol transit and promoting its efflux from macrophages.[139,140]

A unique theory about the pathophysiology of atherosclerosis has gained popularity recently, speculating that established risk factors may serve as a potentiator for an underlying dietary element.[141] Trimethylamine N-oxide (TMAO) is the implicated factor. It is formed from trimethylamine (TMA), which is present in meat, milk, and fish, among other food products obtained from animals. These TMAs are released by gut microbial metabolism, which is crucial for the onset of disease.[142] Following absorption, TMA is transported to the liver, where it is oxidized into the harmful TMAO by flavin-containing mono-oxygenase 3 (FMO3). Studies linking TMAO to the etiology of metabolic and CVDs, such as atherosclerosis, are becoming increasingly numerous.[143] Based on the TMAO hypothesis, only the effects of phloretin, a flavonoid, have been studied in a mouse model of atherosclerosis thus far. Mice were fed a diet rich in choline along with 100, 200, and 400 mg/kg/day of dihydrochalcone phloretin for 10 weeks. This dietary intervention resulted in signs of endothelial dysfunction, elevated oxidative stress, hyperglycemia, and hyperlipidemia. However, treatment with phenyleptin mitigated these harmful consequences, leading to a limitation of weight gain, enhancement of the lipid profile, and maintenance of endothelial and hepatic function.[144]

In a rigorously conducted double-blind crossover study involving 49 healthy male subjects characterized by the *APOE* genotype, quercetin emerged as a compound manifesting discernible antiatherogenic effects. The study spanned a duration of eight weeks during which participants were administered a daily dosage of 150 mg of quercetin. This intervention yielded notable reductions in both waist circumference and postprandial systolic BP across the entire cohort. The investigational outcomes further revealed a salient decline in postprandial triacylglycerol levels, coupled with a concurrent elevation in HDL-cholesterol relative to the placebo group. However, despite these

encouraging findings, endothelial function exhibited no discernible alterations, and a paradoxical slight increase in TNF-α levels was noted in response to quercetin treatment. Importantly, genotype-dependent effects were exclusively discerned in waist circumference and body mass index.[145] The multifaceted impact of quercetin was underscored by its manifestation of several salutary effects on BP and lipid profile. Nevertheless, this positive trajectory was tempered by the observation of mildly proinflammatory actions associated with its administration.

In studies involving enriched foods and complex flavonoid mixtures, a double-blind, randomized controlled trial with 93 postmenopausal women with Type 2 diabetes mellitus investigated the effects of daily administration of 27 g flavonoid-enriched chocolate (90 mg epicatechin + 100 mg isoflavones, aglycone equivalents/day) or placebo. The treatment did not impact intima-media thickness in the carotid artery but did improve pulse pressure variability. Notably, in patients with pulse wave velocity data, a more substantial improvement was observed, corresponding to a 10% reduction in cardiovascular risk. These findings suggested clinically relevant enhancements in arterial stiffness, likely beneficial for atherosclerotic patients experiencing arterial stiffness.[146]

In a randomized, double-blind experiment, a combination of pure anthocyanins was administered to 150 participants diagnosed with hypercholesterolemia, a recognized risk factor for atherosclerosis. The participants received a daily dose of anthocyanins for 24 weeks. Anthocyanins caused serum levels of VCAM-1, C-reactive protein, and plasma IL-1β to drop. Furthermore, following the treatment, there were notable reductions in LDL cholesterol and increases in HDL cholesterol. Additional experiments conducted *in vitro* using different anthocyanins revealed additive or possibly synergistic anti-inflammatory properties.[147] The observed modifications in lipid profiles and inflammatory markers suggest a potential decrease in the risk of atherosclerosis in individuals treated with anthocyanins, particularly those with high cholesterol levels.

6. Conclusion

This review provides a comprehensive examination of the impact of flavonoids on vascular activity, antiplatelet effects, hypertension, and atherosclerosis. The collective evidence presented underscores the multifaceted nature of flavonoids, highlighting their potential as bioactive compounds with significant implications for cardiovascular health. The studies reviewed consistently demonstrate the ability of flavonoids to modulate vascular activity, showcasing their potential to enhance endothelial function and ameliorate factors associated with hypertension and atherosclerosis. The observed antiplatelet effects further contribute to the overall cardiovascular benefits attributed to flavonoids. While the literature consistently suggests a favorable role for flavonoids in cardiovascular health, it is crucial to acknowledge the diversity within this class of compounds and their varied effects across different subclasses and sources. Moreover, genotype-dependent responses, as exemplified in the case of quercetin, add a layer of complexity to the understanding of flavonoid interactions within the cardiovascular system. As research in this field advances, continued exploration into the molecular mechanisms underlying flavonoid actions, their optimal dosages, and potential synergies with other therapeutic interventions is warranted. Such endeavors will contribute to a more nuanced comprehension of flavonoids' role in vascular health and inform the development of targeted strategies for mitigating cardiovascular disorders. In light of the promising findings, flavonoids emerge as promising candidates for further investigation and potential incorporation into preventive and therapeutic approaches for CVDs.

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REVIEW ARTICLE

Prognostic indicators and management of severe acute pancreatitis

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Abstract

Severe acute pancreatitis (SAP) is defined according to clinicobiological, radiological, and evolutionary criteria. In this review, we discuss the main prognostic factors and propose a therapeutic approach based on recent literature. The treatment of SAP primarily involves resuscitation and intensive care, with surgery reserved as a secondary treatment in the event of complications or cases of biliary etiology. Despite advancements, the prognosis remains guarded for severe forms. A critical challenge in this emergency context is the early identification of markers that can guide patients with severe SAP to referent centers with multidisciplinary and specialized teams. These teams play a dual role: providing optimal acute-phase care and ensuring long-term monitoring due to potential functional consequences that may persist post-crisis.

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1. Introduction

Severe acute pancreatitis (SAP) is defined as organ failure and/or local complications such as necrosis, abscess, or pseudocyst, often necrotic,[1] with a mortality rate of 30%. According to the Atlanta Criteria (Table 1), acute pancreatitis (AP) is considered severe in the following situations: (i) When the Ranson or IMRIE scores are >3; (ii) when the APACHE II (Acute Physiology and Chronic Health Examination) score is higher than eight; and (iii) when the C-reactive protein (CRP) level at 48 h is over 150 mg/dl.[2] In addition, some authors consider AP severe if it necessitates hospitalization for at least 3 months or leads to the patient's death.[3]

In 2012, the Atlanta Criteria were revised by a panel of experts, who ruled that AP is classified as severe when it leads to a multivisceral failure syndrome (MVFS) persisting beyond 48 h.[4] Pancreatitis presents as both a medical and surgical emergency, typically affecting adults with an average age of 55 (with extremes ranging from 30 – 70 years old), with a slight predominance of women. Its frequency is difficult to assess: in 1998, France witnessed 16,434 hospitalizations for AP,[5] while the US recorded 250,000 hospitalizations annually for the same reason.[6] Overall, it is estimated that approximately 80% of cases are moderate, with the remaining 20% being severe. Alcohol and biliary lithiasis account

Table 1. Atlanta classification for acute pancreatitis

Stages	Description
Mild	Acute pancreatitis without obvious pancreatic necrosis.
Severe	(i) Visceral failure • Systolic arterial pressure (SAP) < 90 mmHg; • Partial pressure of oxygen (PaO_2) ≤ 60 mmHg; • Creatinine>176.8 µmol/L; • Digestive hemorrhage>500 mL/24 h. (ii) Local complications: Necrosis, pseudocysts, and abscesses. (iii) Ranson score>3. (iv) APACHE II score>8. (v) C-reactive protein (CRP) at 48 h>150 mg/dL.
Necrosis	Presence of a localized or diffuse pancreatic or peripancreatic non-viable zone.

for 90% of SAP. Other causes include hypertriglyceridemia, anatomical abnormalities, drugs, and trauma.[7] However, etiology has no influence on the severity of the condition.

The primary treatment for SAP involves resuscitation and intensive care, with surgery reserved as a secondary treatment in the event of complications. It is also useful in the treatment of biliary etiology. The prognosis remains guarded in severe cases. The overall mortality rate for all forms of AP is 5%, with a breakdown of 3% for moderate cases, 17% for severe cases, and 35% for instances involving infected necrosis, especially when associated with MVFS.[8,9] In the case of infection, mortality varies between 40% and 70%, depending on the study.[10] The implementation of precise algorithms for SAP management, coupled with the constant monitoring of compliance, has enabled Japan to reduce the mortality rate for severe forms to 10.1%.[11] The major challenge in managing this condition is identifying an "ideal marker" early on to predict which patients are likely to develop severe forms so that they can be transferred immediately to a referral center where they will be treated by a trained multidisciplinary team of surgeons, gastroenterologists, and interventional radiologists. To date, such an "ideal marker" has not been identified; therefore, this review aims to analyze available predictive tools and subsequent therapeutic strategies.

2. Diagnosis of severity

2.1. Future prospects

2.1.1. Clinical elements

Generally, clinical presentation is a poor indicator of attack severity. Upon admission, a comprehensive clinical examination detects less than 44% of severe forms.[12] Obesity (with a body mass index >30), age over 70, and chronic alcoholism appear to be the factors that aggravate the condition.[8,13-15] The presence of signs of loco-regional diffusion, such as necrotic spots on the flanks (Grey Turner) or in the periumbilical area (Cullen sign), holds significant prognostic value. However, they are very inconsistent and are found in < 3% of cases of SAP.[16] A rise in intra-abdominal pressure (IAP) is correlated with the severity of the attack; an IAP of more than 14 mmHg is considered a reliable and early marker of a severe form of AP.[17] The MVFS is the most relevant factor in the unfavorable clinical outcome of SAP.[16]

2.1.2. Biochemical factors

A CRP level exceeding 150 mg/L within the first 48 h is indicative of pancreatic necrosis, with sensitivity and specificity both exceeding 80% and an accuracy of 86%.[18,19] However, this assay is of no value in detecting infection with pancreatic necrosis.[20] Similarly, a serum amyloid A level of over 280 mg/L indicates pancreatic necrosis, with a sensitivity of 69% and a specificity of 67%.[21]

On the other hand, procalcitonin is not considered a good indicator of pancreatic necrosis, but it serves as an excellent indicator of infection, whether bacterial or fungal.[22] Regarding the interleukin family, only interleukin 6 is of value in diagnosing severity. Its levels increase in SAP from day 1, peaking at 72 h.[23]

Moreover, granulocyte polymorphonuclear elastase is an early prognostic factor for severity, with a sensitivity of 92%, a specificity of 91%, and an accuracy of 91%. When exceeding a threshold value of 110 µg/L, its positive and negative predictive values are 78% and 96%, respectively, in detecting the severity of the disease.[24,25] Unfortunately, not all of these markers can be detected in time, and their measurement cannot be generalized to all centers. Recently, the measurement of the soluble fraction of E-cadherin in blood has emerged as an early marker of severity that can be used during the first 12 h, thus facilitating improved patient referral.[26]

2.1.3. Clinical-biological scores

The Ranson score (Table 2) assigns a point (01) to each parameter, resulting in a Ranson index ranging from 1 – 11. AP is considered severe when the Ranson index is greater than or equal to three, and mortality approaches 100% when the number of points is greater than or equal to seven. However, the Ranson score cannot be assessed before the 48th h, lacks reproducibility, and is not employed for screening complications.

The Imrie or Glasgow score (Table 3) serves as a practical simplification of the Ranson score, assessing all criteria upon the patient's admission. The APACHE II score (Acute Physiology and Chronic Health Examination) includes criteria such as age, associated defects, hemodynamic, respiratory, and neurological criteria (Glasgow scale). In addition, it involves the assessment of renal, hematological,

Table 2. Ranson criteria for acute pancreatitis

Criteria	Scoring
On admission	
Age	>55 years
Hyperleukocytosis	>16000/mm^3
Blood glucose	>2 g/L (11 mmol/L)
Lactate dehydrogenase	>350 UI/L (1.5 N)
Aspartate aminotransferase	>250 UI/L (6 N)
Within the first 48 h	
Fall in hematocrit	>10
Blood urea	>50 mg/L (8.3 mmol/L)
Blood calcium	< 80 mg/L (2 mmol/L)
PO_2	< 60 torr (mmHg)
Bicarbonate (HCO_3^-) deficiency	>4 mEq/L
Fluid sequestration	>6 L

Table 3. Imrie scoring for acute pancreatitis

Criteria	Scoring
Age	>55 years
Hyperleukocytosis	>15000/mm^3
PO_2	< 60 Torr (mmHg)
Lactate dehydrogenase	>660 UI/L
Blood glucose	>1.80 g/L (10 mmol/L)
Blood urea	>50 mg/L (8.3 mmol/L)
Blood calcium	< 80 mg/L (2 mmol/L)
Blood albumin	< 32 g/L
Aspartate aminotransferase	>100 UI/L

and metabolic function (Table 4). Although it is not specifically designed for biliary pancreatitis (BP), it applies to any severe condition. It can be obtained within the first 24 h and can be repeated regularly to monitor the disease and therapeutic response.[2] In most studies, it has been shown to be superior to the Ranson and Imrie scores in detecting the severity of BP.[27] The APACHE-O score, which correlates with patient obesity by adding two points when the BMI exceeds 30, exhibits an even greater discriminatory value.[28] The combination of the APACHE II with the CRP level demonstrated a better prediction of severity (area under the curve [AUC] = 0.82, specificity = 71%, and sensitivity = 87%) compared to its isolated assessment (AUC = 0.74).

In a recent Japanese study, a new prognostic score for severe pancreatitis was developed, based on three simple criteria assessed on patient admission, with each item scored by one point:
(i) Blood urea ≥ 25 mg/dL;
(ii) Lactate dehydrogenase ≥ 900 UI/L;
(iii) Presence of pancreatic necrosis on computed tomography (CT) scan.

Mortality was 2% for a score of zero, 18% for a score of one, 48% for a score of two, and 67% for a score of three. Similarly, the percentage of infections increased with the score: 2% for a score of 0, 13% for a score of 1, 48% for a score of 2, and 53% for a score of 3. The MVFS was present in 24, 50, 80, and 100% of cases, respectively, corresponding to scores of zero, one, two, or three.[29]

Other authors have explored creating computer software in research protocols, aiming to combine all the clinicobiological data in an artificial neural network to produce a highly reliable prediction of MVFS in SAP.[30,31] For instance, a Swedish team combined just four factors (age, peak creatinine level during the first 72 h, need for mechanical ventilation, and pathological history) to achieve better accuracy (AUC = 0.862) than the APACHE II score (AUC = 0.781) and the Ranson score (AUC = 0.655) in detecting the severity of the condition.[32]

2.1.4. Radiological criteria

In standard radiograph films, the presence of left pleural fluid effusion constitutes an element of diffusion and, therefore, reflects the severity of the disease Conversely, abdominal ultrasound has no role in the diagnosis of severity as the echo of necrotic fat is weak and difficult to distinguish from pancreatic edema, leading to misclassification of the severity of the condition.[33] However, ultrasound plays a greater role in etiological investigation, particularly in detecting biliary lithiasis, with a high sensitivity and specificity approaching 100%.[34,35] Ultrasound should be performed early, as fasting will rapidly induce the formation of vesicular sludge, which falsely increases the rate of SAP attributed to biliary causes.[36] It can also aid in detecting local complications (collections and abscesses) and vascular complications through Doppler ultrasound.

In contemporary practice, the indications for CT scans must be rigorously established, as not all cases of AP warrant this imaging modality. The normal density of the pancreatic gland is between 100 and 150 HU (Hounsfield units). The Balthazar scannographic severity index, which correlates well with morbidity and mortality,[37] is an important diagnostic tool. It is best assessed at the 72nd h, as the injection of contrast medium before this time may aggravate pancreatic necrosis through alteration of the pancreatic microcirculation,[38] and necrosis does not begin to delineate itself until the 72nd h. The CT severity index (CTSI), combining the Balthazar score and the extent of glandular necrosis (Table 5), is also of great prognostic value.

A CTSI score of ≥ 7 points is correlated with a complication rate of approximately 92% and a mortality

Table 4. The Acute Physiology and Chronic Health Evaluation (APACHE) II score items

Physiological variable	Abnormal upper limit				Abnormal lower limit				
	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature (°C)	≥ 41	39 – 40.9		38.5 – 38.9	36 – 38.4	34 – 35.9	32 – 33.9	30 – 31.9	≤ 29.9
Mean arterial pressure (mmHg)	≥ 160	130 – 159	110 – 129		70 – 109		50 – 69		≤ 49
Pulse	≥ 180	140 – 179	110 – 139		70 – 109		50 – 69	40 – 54	≤ 39
Respiratory frequency	≥ 50	35 – 49		25 – 34	12 – 24	10 – 11	6 – 9		≤ 5
Fraction of inspired oxygen (FiO_2) ≥ 0.5 FiO_2 ≤ 0.5	≥ 500	350 – 499	200 – 349		70 – 200	61 – 70		55 – 60	≤ 55
Arterial pH	≥ 7.7	7.7 – 7.69		7.5 – 7.59	7.33 – 7.49		7.25 – 7.32	7.15 – 7.24	≤ 7.15
Natremia (mmol/l)	≥ 180	160 – 179	155 – 159	150 – 154	130 – 149		120 – 129	111 – 119	≤ 110
K blood rate (mmol/l)	≥ 7	6 – 6.9		5.5 – 5.9	3.5 – 5.4	3 – 3.4	2.5 – 2.9		≤ 2.5
Creatininemia (mg/dL) (in the event of renal failure, the score is doubled)	≥ 3.5	2 – 3.4	1.5 – 1.9		0.6 – 1.4		≤ 0.6		
Hematocrit (%)	≥ 60		50 – 59.9	46 – 49.9	30 – 45.9		20 – 29.9		≤ 20
Leukocytes (total/mm^3)	≥ 40		20 – 39.9	15 – 19.9	3 – 14.9		1 – 2.9		≤ 1
15-Glasgow neurological score									
Total acute physiology score									

Notes: The APACHE II score is made up of the sum of total APS, the points for age, and the points for chronic diseases. The allocation of points depending on age is as follows: (i) < 44=0 points; (ii) 45 – 54=2 points; (iii) 55 – 64=3 points; 65 – 74=5 points; and (iv) ≥ 75=6 points. The allocation of points for chronic disease: if the patient has any kind of severe organic insufficiency or immune depression, points should be assigned as follows: (i) In the non-operative context or post-operatively after urgent surgery (5 points) and (ii) post-operatively after elective surgery (2 points).

rate of 17%.[39] In addition, CT analysis incorporates prognostic factors not included in the severity index: ascites, pleural effusion, cephalic location of necrosis, and complications of flows (infection, fistula, pseudoaneurysm, and venous thrombosis).[40] In the same vein, another CTSI based exclusively on the presence of signs of extra-pancreatic diffusion (pleural, retroperitoneal, and ascites), the Epic score (extra-pancreatic inflammation on CT), has been tested. In a pilot study, this new concept enabled the identification of patients likely to develop SAP on the 1st day of hospitalization (AUC = 0.91, sensitivity = 100%, and specificity = 70.8%), pending validation in a larger series. Organized pancreatic necrosis, or "necoma," is a recent and often underestimated entity that represents a zone of post-necrotic pancreatic tissue, commonly mistaken for a recent pseudocyst. This formation results from the liquefaction of pancreatic necrosis, a process that takes 1 – 3 months to manifest. The "necroma" stands out well from the retroperitoneum and can be easily removed by surgery. The density of this ovoid lesion is greater than 20 HU, distinguishing it from the recent pseudocysts, whose density does not exceed 10 HU.[2]

Magnetic resonance imaging (MRI) and cholangio-MRI are employed when CT scans are contraindicated (for allergic or other reasons). They are more appropriate in countries where pancreatitis is predominantly of biliary origin (North Africa and southern Europe), as they are more reliable than CT in detecting choledocholiths or pancreatic calculi.[41] Cholangio-MRI, with its ability to map the biliary-pancreatic tree, should be used in the etiological investigation of so-called idiopathic pancreatitis to identify anatomical anomalies or early cancers of the biliary-pancreatic junction. MRI is believed to have a greater prognostic value than CT in these cases.

All these elements have recently converged into a Japanese radio-biological severity classification. This new classification integrates eight biological items, the patient's age, and scannographic data. The calculation is based on the sum of these elements (Table 6). When the score is < 3, mortality is only 0.7%. Between 3 – 6, mortality rises to 12.5%, and >6 points, mortality reaches 31% with an AUC of 0.86.[42]

2.2. Retrospective elements (complications and death)

2.2.1. Local complications

Superinfection of necrosis occurs in 40 – 70% of cases, where necrotic tissue becomes infected with germs similar to the flora of the digestive tract (enterococci and enterobacteria), translocating from the colon. The earlier the necrosis infection, the higher the mortality rate (with a peak in the 3rd week). Despite recent studies showing a decrease in infection rates to 10 – 40% of cases, mortality

Table 5. Balthazard score and severity indices

Stages	Points	% Necrosis	Extra points	Severity index	Clinical impact
A: normal pancreas	0	0	0	0	0% abscesses
B: localized or generalized hypertrophy to the gland	1	0	0	1	Idem at stage A
C: heterogeneous gland with densification of the peripancreatic fat	2	< 30	2	4	12% abscesses
D: greasy flow in a single space (extra-pancreatic collection)	3	30 – 50	4	7	17% abscesses; 8% of death
E: extra-pancreatic greasy flow or presence of gas in the intra-pancreatic or extra-pancreatic area	4	>50	6	10	61% abscesses; 17% of death

Table 6. Assessment of severity of acute pancreatitis according to new prognostic factors and computed tomography grading (Japanese classification)

Prognostic factors (1 point for each factor)	
1.	Base excess ≤ 3 mEq/L or shock (systolic blood pressure < 80 mmHg)
2.	PaO_2 ≤ 60 mmHg (room air) or respiratory failure (respiratory management is needed)
3.	Blood urea nitrogen ≥ 40 mg/dL (or creatinine [Cr] ≥ 2.0 mg/dL) or oliguria (daily urine output < 400 mL even after intravenous fluid resuscitation)
4.	Lactate dehydrogenase ≥ 2 times of upper limit of normal
5.	Platelet count ≤ 100000/mm^3
6.	Serum calcium ≤ 7.5 mg/dL
7.	C-reactive protein (CRP) ≥ 15 mg/dL
8.	Number of positive measures in systemic inflammatory response syndrome criteria ≥ 3
9.	Age ≥ 70 years
CT grade by contrast-enhanced CT	
1.	Extrapancreatic progression of inflammation
	Anterior pararenal space: 0 point
	Root of mesocolon: 1 point
	Beyond the lower pole of the kidney: 2 points
2.	Hypoenhanced lesion of the pancreas The pancreas is conveniently divided into three segments (head, body, and tail)
	Localized in each segment or only surrounding the pancreas: 0 point
	Covers two segments: 1 point
	Occupies entire two segments or more: 2 points
Total score: 1+2	Total score=0 or 1 (Grade 1)
	Total score=2 (Grade 2)
	Total score=3 or more (Grade 3)

Notes: For computed tomography (CT) grade determined using contrast-enhanced CT: (i) If prognostic factors are scored as 3 points or more, or (ii) if CT grade is judged as Grade 2 or more, the severity grading is evaluated to be as "severe."

remains at 100% if no therapeutic intervention is taken in the infected necrosis.[43] The natural course of pancreatic juice diffusion can lead to the formation of pseudocysts and fistulization into hollow organs. Consequently, a formidable complication is represented by hemorrhage, often resulting from fissuring or rupture of an aneurysm of the gastro-duodenal or coronary stomachic (left gastric) arteries. Arterial bleeding responds well to embolization, while venous bleeding is more difficult to control and may require pancreatic or even splenopancreatic resection to ensure hemostasis.[2] Stress-induced digestive hemorrhages or diffuse hemorrhage due to coagulation disorders may also occur in this context.

2.2.2. General complications

Once enzymopathy becomes systemic, all organs may be affected (cardiovascular, pleuropulmonary, renal, and neurological systems). In metabolic terms, carbohydrate intolerance is a poor prognostic criterion.[44] In SAP, 50% of deaths occur during the 1st week.[44]

3. Treatment

3.1. Goals

Once the severity of the condition has been diagnosed and the patient has been appropriately referred, the aims of treatment are to control pain and shock and to restore electrolyte and metabolic balance. Surgery is required for the majority of septic complications and for some complications of sterile necrosis. All curable etiologies must be properly managed to avoid recurrence.

3.2. Resources

3.2.1. Medical

The management of pain in pancreatitis typically involves the administration of analgesics in successive stages with potent options (morphine) such as Dilaudid tablets (hydromorphone hydrochloride), often being necessary due to the severity of the pain.[45] Utilizing a morphine pump makes a definite contribution and allows optimal, more personalized pain management with a grade 1C recommendation. If there is no response, multi-modal analgesia may be used, including the use of an epidural.[46] However, in cases of biliary pancreatitis, pethidine is preferred over morphine due to its lower propensity for causing ODDI sphincter spasm.[47]

Intensive care represents the cornerstone of treatment. Aggressive crystalloid resuscitation must be used to correct hydroelectrolyte disorders. Central catheterization is strongly recommended, to monitor central venous pressure on the one hand and to adjust filling to avoid any overload on the other hand.

In cardiac patients, a Swann-Ganz or pulmonary artery catheter is strongly recommended. Yan *et al.*[48] exhibited in their study of SAP patients that CAP-guided hydroelectrolytic resuscitation reduced the length of stay in the intensive medical care unit. In the same group of patients, there was a decreased need for renal dialysis and a lower rate of MVFS. However, there was no significant difference in mortality. In cases of refractory shock, vasoactive drugs should be employed, while strict monitoring and maintenance of respiratory and renal functions are essential.

A bladder catheter is utilized in conjunction with IAP monitoring. Insulin therapy should be introduced early in cases of impaired glycemic regulation because when it prevents blood glucose levels from exceeding 110 mg/dL, mortality is reduced by 3 – 4%.[49] Stress-induced gastrointestinal hemorrhage can occur, especially when the pH of the gastric aspiration fluid is below three. This condition should be prevented through the administration of proton pump inhibitors.[47] In addition; preventive heparin therapy should be instituted.

Caloric intake is provided parenterally during the algic phase, but as soon as the pain subsides (especially after food ingestion), progressive enteral feeding can be introduced. The aims of early enteral feeding are to modulate the systemic inflammatory response and reduce bacterial translocation and pancreatic infection by maintaining normal digestive flora and stimulating intestinal peristalsis by inserting a naso-jejunal tube. A recent meta-analysis of seven randomized trials concluded that enteral nutrition in AP is correlated with a reduction in infection, morbidity, visceral failure, and length of hospitalization.[50] Several studies have reported that enteral nutrition reduces the rate of pancreatic infection and multi-visceral failure. Furthermore, enteral feeding is correlated with a lower cost and a lower risk of nosocomial infections.[51,52] Finally, mortality in SAP does not differ between parenteral and enteral feeding.

Over 80% of deaths in AP are caused by septic complications due to the bacterial infection of pancreatic necrosis.[53] Patients with retro-pancreatic necrosis are more prone to bacterial infection.[54] Logically, antibiotic therapy should only be administered in the event of superinfection of the necrosis documented by bacteriological sampling (either by blood culture or by fine needle puncture of the abscessed collection with a specificity of 100% and a sensitivity over 90%), and it should be adapted to the germ according to the results of the antibiogram. Where appropriate and when the bacteriological proof is unavailable, probabilistic antibiotic therapy with carbapenems is recommended when signs of superinfection are present (fever, hyperleukocytosis, hemodynamic instability, gas on imaging, associated angiocholitis, or extra-pancreatic infection confirmed by fine needle aspiration).[53,55,56] Three published meta-analyses have compared the administration of antibiotic prophylaxis to control groups.[55,57,58] Each study reported a reduction in the severity of the condition and in mortality following the use of antibiotics. The Cochrane Foundation recently published the results of five studies involving 294 patients, who also support a mortality reduction following the use of antibiotic prophylaxis in PAS.[59] Despite a lack of consensus, due to its potential to improve certain septic states, we recommend the use of antibiotic prophylaxis in suspected or confirmed necrosis, regardless of infected status. However, in 30% of cases, untimely antibiotic therapy leads to superinfection of the necrosis by *Candida albicans*, complicating treatment and prognosis.[60] Antibiotic therapy should not exceed 14 days. If infectious signs persist beyond this period, empirical anti-fungal therapy should be introduced. He *et al.*[61] demonstrated a reduction in colonization after antifungal agent use in a study involving 70 patients, though no mortality reduction was observed.

Agents targeting pancreatic secretion, including trypsin inhibitor aprotinin, platelet activator factor (PAF) inhibitor, protease inhibitor (gabexate mesylate), and octreotide, have shown no benefit, according to a recent meta-analysis.[50] However, lexipafant (a PAF inhibitor) is thought to reduce the disintegration of the intestinal mucosa, thereby suppressing systemic inflammatory response syndrome. Two randomized trials have demonstrated its involvement in reducing the rate of sepsis and multivisceral failure, but it has no effect on mortality.[62,63]

3.2.2. Radio-guided and endoscopic therapeutic procedures

Peritoneal dialysis by puncture-washing has shown its efficacy on cardio-respiratory function but without any effect on reducing mortality. The radiation-guided puncture of infected collections or pseudocysts using large-bore drains (24 Charrière) gave very good results in a French study (100% survival in pancreatic abscesses), provided that irrigation was associated.[64] The disadvantage of this approach is the appearance of a pancreatic fistula. Drainage has, above all, a temporizing role by avoiding recourse to surgery in the acute phase because delayed surgical treatment of well-limited lesions allows *en bloc* necrosectomy to be performed, removing all the necrotic debris and thus avoiding repeat operations.

When a hemorrhage or an aneurysm is detected, an embolization can be used to stop it. Puncture under echo-endoscopy is particularly recommended when the pseudocyst is retro-gastric and after 4 weeks to achieve a certain "maturity" of the collection.[45] The placement of a stent allows for internal drainage of the collection. The endoscopic sphincterotomy (ES) is very useful in severe obstructive biliary pancreatitis in the acute phase (first 72 h) with a grade 1A recommendation.[45,51] This procedure may improve the prognosis, but the latter remains dependent, above all, on the extent and infection of the necrosis.[65-68] Candidates for ES should be selected by echo-endoscopy or cholangio-MRI in order to limit unnecessary ones without choledocholithic or oddial obstruction. Performing an endoscopic retrograde cholangiopancreatography (ERCP) for biliary pancreatitis where the obstruction has not been confirmed would unnecessarily increase the risk of infection, especially fungal infection.[69] Furthermore, ES carried inherent risks, with a mortality rate of 2% and a morbidity rate of 8%. In the study by Boicean *et al.*[70] involving 134 patients who performed ERCP to extract choledochal lithiasis (n = 48 with post-ERCP pancreatitis [PEP] and n = 86 without PEP), a higher risk of PEP was observed in female subjects and lower risk in patients who underwent main bile duct clearance with the Dormia probe and dilatation balloon (odds ratio [OR] = 2.893; confidence interval [CI] 95% = 1.371 – 6.105; P = 0.005 and, respectively OR: 0.346; CI 95% = 0.156 – 0.765; P = 0.009), without biliary stent placement. In addition, the data analysis provided new research evidence, demonstrating that higher values of CRPR-CRP after ERCP/CRP on admission: OR = 4.337; CI 95% = 1.945 – 9.668; $P < 0.001$; total bilirubin inverted ratio: OR = 4.004; CI 95% = 1.664 – 9.634; P = 0.002; and neutrophil lymphocyte ratio: (OR = 3.281; CI 95% = 1.490 – 7.221; P = 0.003) predicted the occurrence of PEP.[70]

3.2.3. Surgery

Surgery should be postponed until at least the 30th day, a timeline adopted by the majority of international pancreatology societies.[71] This waiting period is associated with lower morbidity and mortality.[71-75] This attitude allows a patient to be operated on after stabilization of the systemic inflammatory reaction and after demarcation of the necrotic lesions from the healthy parenchyma, which is a valuable aid for the surgeon. The current best indicator of the time for surgery is the increase in the APACHE II score during the intensive care period. In general, the indication for surgery is in three situations (Table 7 and Figure 1):

(i) In a rare group of patients, early intervention is necessary when their state of health deteriorates despite aggressive care;[71] this may be due to massive hemorrhage or perforation. Early surgery is also indicated in cases of compartment syndrome, which complicates up to 80% of PAS.[75-77] When the IAP exceeds 25 mmHg, decompression by laparotomy is indicated, which improves hemodynamic status as well as respiratory and renal function.
(ii) Infected pancreatic necrosis;
(iii) Certain complications of sterile necrosis may lead to surgery. Firstly, in the event of sterile necrosis exceeding 50% of the pancreatic area and requiring assisted ventilation or hemodialysis, surgery is indicated. It may also be persistent AP,[78] or AP due to refeeding.[79] Ductal interruption syndrome is often associated with these conditions and is thought to result from necrotic rupture of the pancreatic duct, leading to retention of pancreatic juice in the distal part of the gland. This anatomical condition responds well to a pancreatico-jejunal anastomosis on a Y loop; otherwise, there is a risk of transitioning to chronicity.[80] Generally speaking, however, in cases of sterile necrosis, the results obtained with a conservative therapeutic strategy are comparable to those obtained with a surgical approach; 30 to 60% of SAP with sterile necrosis are cured without recourse to surgery.[54,81] Furthermore, mortality in cases where sterile necrosis is surgically debrided is 12% to 21%, and when it is treated non-

Table 7. Principles of surgical management of acute pancreatitis

Atlanta Classification	Therapeutic principles
Mild acute pancreatitis (edematous pancreatitis)	• No surgery other than cholecystectomy during the same hospital stay
Severe acute pancreatitis	
Sterile necrosis	• No systematic surgery • Surgery is indicated if there is no response to intensive care (when necrosis exceeds half the pancreatic area), ductal interruption syndrome, recurrent pancreatitis, or refeeding one
Infected necrosis	• Mini-invasive surgical debridement (VARD) + continuous lavage (step-up approach) or open packing (pancreaticostomy chimney) in the event of failure (step-down approach)
Pancreatic abscess	• Echo or scan-guided drainage • Surgical drainage in cases of persistent sepsis
Acute pseudocyst	• Drainage by interventional radiology or surgery depends on the resources available to the hospital center

Abbreviation: VARD: Video-assisted retroperitoneal drainage.

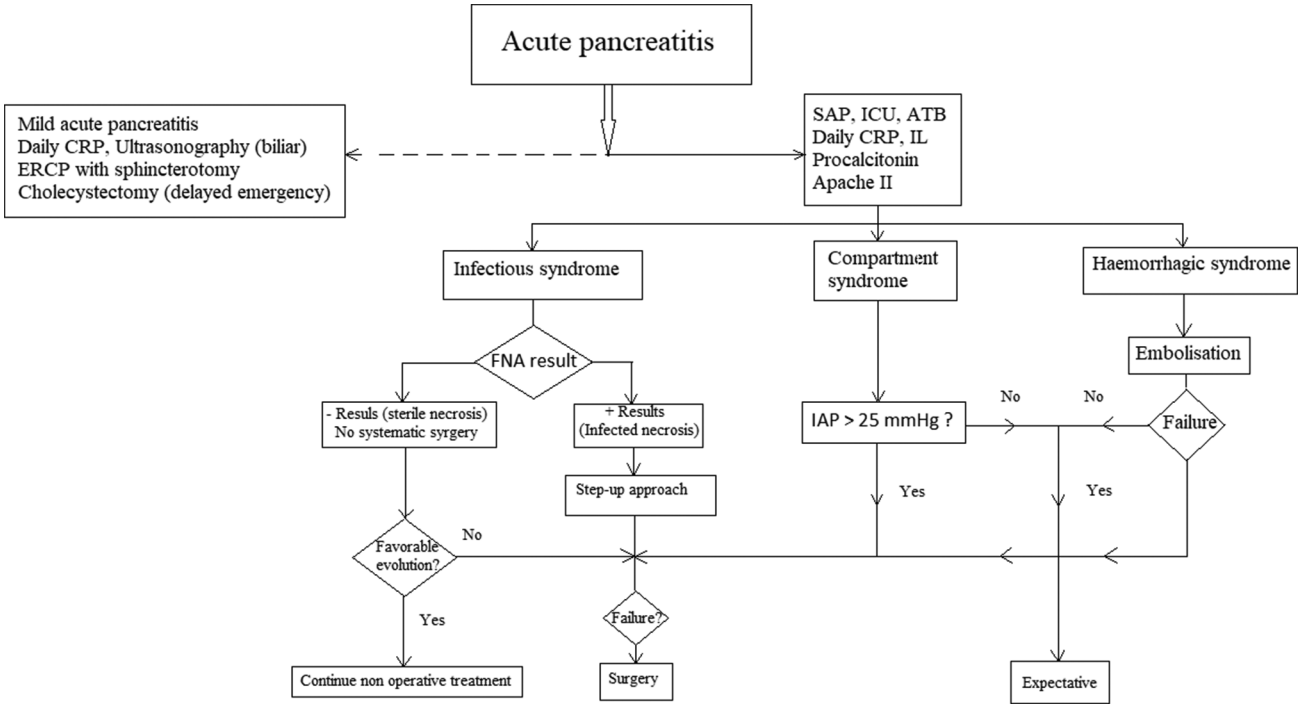

Figure 1. Decision-making tree for acute pancreatitis. Figure created using the Microsoft Paint software.
Abbreviations: AP: Acute pancreatitis; APACHE II: Acute Physiology and Chronic Health Examination II; ATB: Antibiotics; CRP: C-reactive protein; ERCP: Endoscopic retrograde cholangiopancreatography; FNA: Fine needle aspiration; IAP: Intra-abdominal pressure; ICU: Intensive care unit; IL: Interleukin; SAP: Severe acute pancreatitis.

operatively, it is only 2 – 5%.[51,81] A retrospective study analyzing changes in surgical attitude between two periods (period A: 1982 – 1993 and period B: 1993 – 2001) reported the following results:[82] There was a high rate of surgical intervention for sterile necrosis and fewer interventions for infected necrosis during period A. In period B, the opposite was observed.

During period B, there was less bacterial infection (*Escherichia coli*) and more fungal infection, compared with period A. During the second period, there was more mortality in the group with sterile necrosis, and a stabilization of this rate in the group with infected necrosis. In both periods, more patients with sterile necrosis underwent mechanical ventilation than those with infected

necrosis (51% vs. 35%). Sterile necrosis was operated on earlier (1st week) than infected necrosis (3rd week).

All this shows that there is a growing trend toward a non-operative approach to SAP with sterile necrosis. The surgical strategy is based on two approaches: (i) A necrosectomy followed by drainage (the so-called closed or step-up approach). Video-assisted retroperitoneal drainage (VARD) is used, with a direct approach to the retroperitoneum without contamination of the peritoneal cavity. (ii) Debridement followed by abdominal packing or a pancreatectomy chimney (open technique or step-down approach). This technique is generally used when the closed technique has failed.

A meta-analysis comparing the two techniques concluded that the open technique is associated with greater morbidity (incisional hernias) and a possible increase in the mortality rate,[47] while the closed technique reduces the rate of post-operative complications.[82]

The operation is generally performed through a bi-subcostal approach. More recently, the retroperitoneal[83,84] and laparoscopic[85-87] approaches have been used with acceptable results in selected cases. The retroperitoneum is penetrated after opening the lesser sac. Debridement is performed with a finger and is stopped as soon as the pancreatic tissue starts to bleed, which attests to its vitality. Extension of the necrosis to the para-colic and mesenteric fat must also be gently debrided. If all the necrotic material has been removed, the abdomen can be closed with suction drains: on the left, the drain is positioned next to the left colonic angle, under the lower splenic pole; on the right, the drain is slid into the subhepatic space.

In the case of extensive extra-pancreatic necrosis, other drains must be placed opposite the debrided areas. The gastrocolic and duodeno-colic ligaments are sutured, and hypertonic saline lavage is started at a rate of 2 L/h. When the septic signs improve, the drainage volume is reduced, and when no necrotic debris is recovered, the drains are removed. However, when the necrosectomy is incomplete, a pancreaticostomy tube should be left in place to allow the pancreatic cavity to communicate with the outside environment through a Mikulicz bag in anticipation of iterative necrosectomies. However, the necrosectomy is not without risk. Surgical necrosectomy is often followed by re-accumulation of peripancreatic fluids, but these collections can be drained percutaneously. Pancreatic or enterocutaneous fistulae and parietal complications (sepsis, evisceration) may occur.[10] Bleeding is rare, and can be managed by arterial embolization. Non-surgical complications of necrosectomy include impaired renal function when performed during the inflammatory phase, and exocrine and endocrine pancreatic insufficiency.[20]

Surgery has a place in the treatment of etiologies, particularly biliary, by removing the reservoir of stones by cholecystectomy,[88] with more or less exploration of the bile ducts and their drainage after the complete evacuation of the stones that have migrated to their level. This treatment is carried out laparoscopically. In SAP, cholecystectomy should be performed within 4 – 6 weeks post-critical with a 2C guard of the recommendation.[45] When the gallbladder is left in place after an episode of acute biliary pancreatitis, 80% of patients will have recurrent episodes of pancreatitis within a year.[88]

4. Long-term future

4.1. Recurrences and progression to chronicity

Riaz *et al.*[89] have demonstrated that multiple recurrent episodes of AP lead to chronicity. Notably, recurrent episodes are more frequent in ethylic patients,[32,90] affecting up to 32% of cases. Nordback *et al.*[90] reported a recurrence rate of 46% among 568 patients with alcoholic AP, and 80% of recurrences occurred within 4 years of the first episode. This underscores the importance of management following an alcoholic AP. In addition, a Japanese study demonstrated that the rate of recurrence in patients with pancreatic necrosis was higher than in patients without necrosis (32% vs. 5%).[91] In the same study, the prognostic factors that exhibited a significant difference in recurrence were hyperleukocytosis and CRP levels. Similarly, the rate of transition to chronicity was higher in patients with necrosis than in patients without necrosis (30% vs. 13%). The prognostic factors favoring this transition are a high white blood cell count, a fall in hematocrit, and base excess (BE).

4.2. Development of diabetes mellitus (DM)

The factors that correlate with the development of DM in PAS are blood glucose and BE on admission. There appears to be no relationship between the extent of necrosis and the development of DM.[92] Alcoholic SAP is correlated with a significant increase in the rate of DM compared with biliary SAP.[3] The highest rate of DM was noted in patients who have undergone pancreatic resection after an episode of SAP; depending on the study, this rate varies between 54% and 90% of cases.[92-94]

4.3. Impairment of exocrine function

Early exocrine pancreatic insufficiency is a common denominator after an episode of SAP. In the case of SAP, this failure correlates with the degree of extension of pancreatic necrosis.[94] Exocrine function tests are disturbed in 10 – 75% of patients 1 month after an episode of SAP. Recovery is not the general rule.[95,96]

5. Conclusion

SAP remains a serious pathology, despite advancements in therapeutics and resuscitation techniques. It is primarily a medical emergency and ideally necessitates management in an intensive care unit under the supervision of a multidisciplinary team. Surgical intervention is typically reserved for cases involving local complications, mainly those of a septic nature, as well as for the treatment of biliary etiology. Prevention of nosocomial infection must be an integral part of the therapeutic strategy since some studies have reported a mortality rate of 28.4% due to infectious hospitalism in SAP cases.[97] Following an episode of SAP, the outcome is sometimes marked by the appearance of functional sequelae, a phenomenon increasingly recognized and more prevalent among patients who have undergone surgery.[3] Other factors predictive of these sequelae should be investigated, and long-term monitoring of patients with such outcomes is essential.

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The authors declare that they have no competing interests.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Hesperetin alleviates pulmonary injury in a blunt chest trauma-induced pulmonary contusion model in rats

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Abstract

Pulmonary contusion (PC), a condition that occurs frequently in severe thoracic injuries, is a significant contributor to mortality in those under the age of 40. Hesperetin, a natural flavonoid derivative of hesperidin, is a substance found in various citrus fruits such as oranges and grapefruits and possesses a variety of biological activities, including antiapoptotic, antioxidant, and anticancer effects. In the current study, we investigated the effect of hesperetin on pulmonary tissue structure, expression of some pro-inflammatory cytokines, and mediators in a PC model induced by blunt chest trauma. In this study, 18 adult male Wistar albino rats (8 – 10 weeks, 250 – 300 g) were used. The rats were divided into three groups: control, PC, and PC + hesperetin. Hesperetin administration (100 mg/kg/day oral) was completed for 7 days following induction of the model. The wet/dry weight ratio of pulmonary tissue was determined. Tumor necrosis factor alpha (TNFα) and malondialdehyde (MDA) in lung tissues, serum interleukin (IL)-6, and IL-1β levels were determined using the enzyme-linked immunosorbent assays. Pulmonary tissue specimens were examined histologically using hematoxylin-eosin and Masson trichrome staining. Inducible nitric oxide synthase (iNOS) activity was determined using immunohistochemical methods. Hesperetin administration reduced TNFα and iNOS activity, serum IL-1β, IL-6, MDA, and wet/dry weight ratio in pulmonary tissue to improve pulmonary function. Our results showed that administration of hesperetin prevents activation of local inflammatory mediators, thereby obstructing the proinflammatory cytokine cascade and tissue injury.

Keywords: Trauma; Tumor necrosis factor alpha; Malondialdehyde; Interleukin 6

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1. Introduction

Pulmonary contusion (PC) is a common injury due to blunt chest trauma observed in 30 – 75% of patients attending emergency medical and trauma centers.[1] In particular, high rates of PC were observed after the occurrence of chest injuries in pediatric patients.[2] Regardless of the severity, estimated rates of mortality attributable to PC range from 14% to 40%. In addition, it has been reported as the direct cause of death in 20 – 25% of patients with multiple trauma.[3] PC may trigger acute pulmonary injury, pneumonia, and respiratory failure syndromes.[4] In addition, hypoxia caused by PC may affect functions of distant organ such as kidneys, brain, and liver.[5] Although the definite mechanisms of PC have not been fully clarified, factors such as increased pulmonary permeability and edema, inflammation, and ventilation/perfusion incompatibility have been reported to play roles in its pathophysiology.[6,7] While there is no specific pharmacological treatment for PC, symptomatic and supportive treatment are applicable to the affected patients.[6]

PC may develop along with abnormal inflammatory response and heightened oxidative stress following blunt chest trauma.[2] Chest trauma can trigger the activation of crucial transcription factors, such as nuclear factor kappa beta, which have a pivotal role in inflammation and the transcription of proinflammatory genes, leading to the activation and synthesis of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and inducible nitric oxide synthase (iNOS).[7] The pulmonary inflammation and tissue injury occurring with PC has been reported to be associated with increased pro-inflammatory cytokine levels in many studies.[8,9]

Experimental investigations have found that certain pharmacological compounds with anti-inflammatory and antioxidative properties can suppress pro-inflammatory cytokine production and oxidative stress, alleviating tissue damage caused by PC and improving clinical symptoms.[6,10,11] Hesperetin is a natural bioflavonoid derivative synthesized after the hydrolysis of hesperidin. Among its many pharmacological effects, it is known to be antioxidant, anti-inflammatory, antiapoptotic, antitumor, and antihyperlipidemic.[12] Administration of hesperetin has been reported to support anti-inflammatory effects and act as a strong free radical scavenger in many *in vivo* and *in vitro* experimental studies.[13,14] In a lipopolysaccharide (LPS)-induced pulmonary injury model, hesperetin administration was found to reduce tumor necrosis factor alpha (TNF-α) and serum IL-6 levels and protect tissue structure.[15] However, the protective role of hesperetin therapy in the model of blunt chest trauma-induced PC has not yet been investigated. Therefore, we aimed to investigate the effect of hesperetin administration on pulmonary tissue architecture and immunohistochemical expression of pulmonary iNOS. Further, we examined the effect of hesperetin on serum inflammatory markers such as IL-1β, IL-6, and TNF-α and the oxidative stress markers such as superoxide dismutase (SOD), malondialdehyde (MDA), and catalase (CAT) enzyme levels in a rat PC model.

2. Materials and methods

2.1. Chemicals

The hesperetin used in this research was obtained from Santa Cruz (Santa Cruz Biotech, Dallas, TX). Hematoxylin and eosin were obtained from Merck (Germany). Ketamine was purchased from Pfizer (İstanbul, Turkey) and xylazine was from Bayer (Turkiye). iNOS primary antibodies were purchased by Novus Biologicals (Littleton, USA). IL-1β, IL-6, and TNF-α ELISA kits were obtained from Shanghai Biotech (Shanghai YL Biotech Co., Shanghai, China). The MDA kit was acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Secondary antibody kits used for immunohistochemical labeling (Thermo Scientific, Fremont, CA, USA) and AEC (3-amino-9-ethylcarbazole) used as chromogen were obtained from Thermo Scientific (USA).

2.2. Animals

Animals used in this study were obtained from Tekirdağ Namık Kemal University Experimental Animals Research and Implementation Center (NKUDAM). Eighteen male Wistar albino rats (250 – 300 g, 8 – 10 weeks old) were used in this study. During the study duration, animals were housed at NKUDAM under standard conditions (22 ± 2°C temperature, 50 – 60% humidity, and 12-h light/12-h dark cycle) and fed standard pellet feed and water *ad libitum*.

2.3. Ethical approval

All procedures in this study were approved by Tekirdağ Namık Kemal University Experimental Animals Local Ethics Committee. All experimental procedures in the study were completed in accordance with the Care and Use of Laboratory Animals Guidelines recommended by the National Institutes of Health.

2.4. Experimental procedures

The rats used in this study were divided into three groups (n = 6 each): control, PC, and PC + hesperetin group. No intervention was performed to the control group. PC induction was executed according to a previously reported method.[16] The right hemithorax of each rat under anesthesia (ketamine-xylazine; 50 – 15 mg/kg, ip), which

was laid in ventrodorsal position, was thumped with a cylindrical object weighing 0.4 kg dropped from 0.5 m above. Thus, the chest cage of the rat received an energy amounting to 1.96 J, measured according to the formula:

$$E = m \times g \times h \quad (I)$$

where E = Energy, m = Mass of cylindrical object (0.4 kg), g = Gravity (9.8 m/s^2), and h = Height (0.5 m). Animals in the PC + hesperetin group were administered hesperetin for 7 days with the aid of intragastric gavage at a dose of 100 mg/kg/day dissolved in 1 mL physiological saline. At the end of the 7th day, the rats were opened on the midline under anesthesia (ketamine-xylazine; 90 – 15 mg/kg, ip), and blood samples were taken by cardiac puncture and pulmonary tissue was removed for future analyses.

2.5. Determination of lung wet/dry weight ratio

The left superior lobe of the lungs was used to assess pulmonary edema. The fresh tissue was washed with phosphate-buffered saline (PBS), dried, and weighed. The wet/dry weight ratio was calculated according to a previous study by weighing the tissues again after being kept at 80°C for 24 h.[17]

2.6. Histopathological analysis

The left middle lobe was immersed in a 10% buffered neutral formalin solution for fixation for 48 h before hematoxylin-eosin (H&E) and Masson trichrome staining. After fixation, tissue samples were soaked in tap water overnight and then processed in a series of routine procedures before being submerged in paraffin. Paraffin blocks were cut to sections with 5 μm thickness using a rotary microtome (Slee, MPS, Germany) and then subjected to H&E staining, Masson trichrome staining, and immunohistochemical iNOS labeling. H&E and Masson trichrome staining were conducted according to steps described elsewhere.[7] H&E-stained slides were rated by two histopathologists, who were blinded to the study groups, for histopathological changes, interalveolar edema, interalveolar hemorrhage, and neutrophil infiltration using a range of grades from 0 to 4 (0: none; 1: mild; 2: moderate; 3: severe; 4: overwhelming) with total maximum score of 12.[18] The distribution of connective tissue in pulmonary tissue and alveolar wall thickening was confirmed with Masson trichrome staining. Microscopic examination was performed with an Olympus CX41 (Olympus, Tokyo, Japan) and image analysis system (Kameram Gen III, Argenit, Istanbul, Turkey) software.

2.7. Immunohistochemical iNOS labeling

Immunohistochemical iNOS labeling was conducted using the avidin-biotin complex peroxidase technique. Deparaffinized pulmonary tissue sections were dehydrated with ethanol, washed in PBS, placed in a citrate buffer to reacquire antigens, and then heated in a microwave (Arcelik, MD550) for 5 min. After cooling down, the sections were treated with 3% hydrogen peroxide to suppress endogenous peroxides. Tissue incubation with primary antibody was performed in a humidified chamber for over 1 h at 37°C. Each tissue section was incubated with streptavidin-biotin-labeled antibodies for 30 min, and subsequently, each of them was labeled with AEC. Contrast staining was completed using Mayer's hematoxylin. The iNOS-positive cells in the pulmonary tissues were enumerated per squared millimeter (mm^2).[7]

2.8. ELISA analysis

Pulmonary MDA levels (nmol/mg protein) were determined using a commercial kit according to the manufacturer's instructions. Commercial ELISA kits were employed to measure the pulmonary levels of MDA (catalog no: CK-E10376; Eastbiopharm, Hangzhou, China) and TNF-α (KRC3011, Thermo Scientific), as well as the serum levels of IL-1β (BMS630, Thermo Scientific) and IL-6 (BMS625, Thermo Scientific), according to the manufacturers' instructions. The absorbance of the tested samples was measured at 450nm using Multiscan Go Spectrophotometer (Thermo Fisher Scientific, MA, USA). The levels of MDA and proinflammatory cytokines were determined with the help of standard curves.

2.9. Statistical analysis

Data are expressed as mean ± standard deviation in this paper. The Kruskal–Wallis test was used to evaluate the data, and Mann–Whitney *U* test was employed to analyze the differences among groups. All statistical analyses were performed using the PASW statistic 18.0 (SPSS Inc, Chicago, IL, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Histopathological findings and lung wet/dry weight ratio

Histopathological investigation of lung tissue was performed using H&E and Masson trichrome staining (Figure 1). The H&E staining revealed that the lung tissue structure in the control group was normal. In the PC group, alveolar wall thickening, alveolar hemorrhage, alveolar edema, and increased leukocyte infiltration were observed. In the PC + hesperetin group, these histopathological changes were also present but at a milder degree. In addition, milder alveolar thickening and edema in the PC + hesperetin group were confirmed with Masson

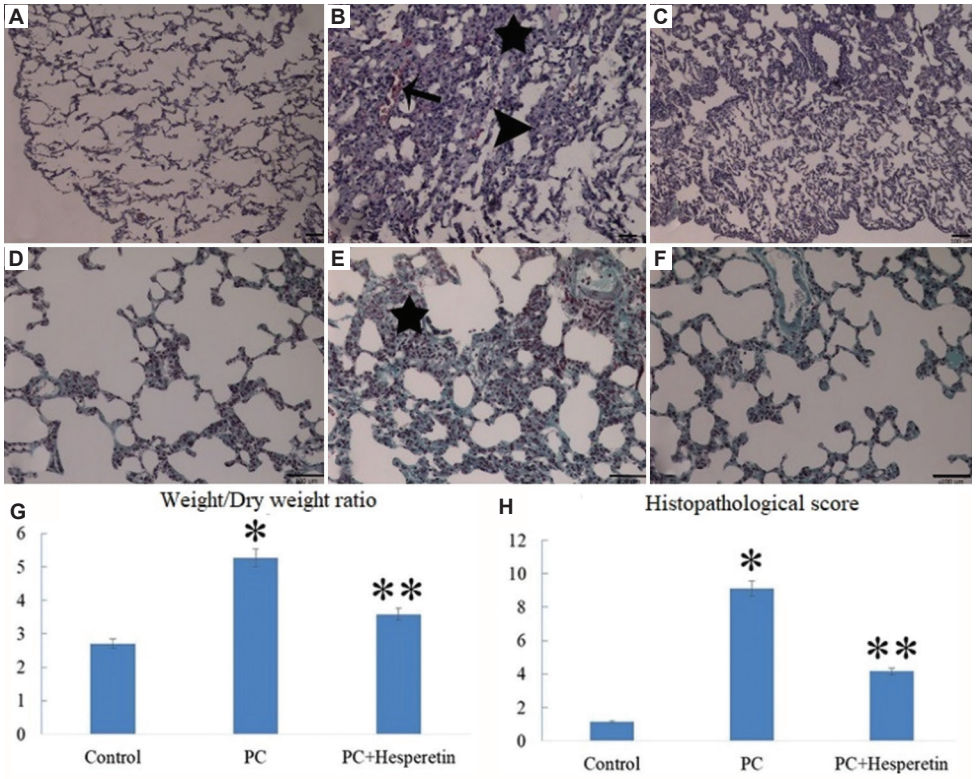

Figure 1. Histopathological examination of tissues from blunt chest trauma-induced PC rats treated with hesperetin. (A-C) H&E-stained lung sections: control (A), PC (B), and PC+hesperetin (C). (D-F) Masson's trichrome-stained lung sections: control (D), PC (E), and PC+hesperetin (F). (G) Comparison of histopathological score between groups. (H) Comparison of wet/dry weight ratio between groups. For panels (A) to (F): Scale bar = 100 μm; arrow denotes pulmonary hemorrhage; arrow head denotes edema; star denotes pulmonary infiltration; sections were observed microscopically with ×100 magnification (A-C) and ×200 magnification (D-F). For panels (G) and (H): *P<0.05 compared to control group; **P<0.05 compared to PC group. Abbreviation: PC: Pulmonary contusion.

trichrome staining. These assessments confirmed that hesperetin administration led to a significant reduction in histopathological change scores compared to the PC group (P = 0.002) (Figure 1G). In parallel with this, hesperetin administration was determined to cause a statistically significant reduction in the wet/dry weight ratio compared to the PC group (P = 0.005) (Figure 1H).

3.2. Immunohistochemical analyses of iNOS

The control group displayed no positive expression of iNOS, as determined by immunohistochemical means (Figure 2A). Increased iNOS expression concomitant with increased inflammatory cell infiltration was observed in alveolar epithelial cells and inflammatory areas in the PC group (Figure 2B). In the PC + hesperetin group, iNOS immunoreactivity was observed to be reduced compared to the PC group (P = 0.002) (Figure 2C). The mean iNOS-positive cell counts for the three groups are given in Figure 2D.

3.3. MDA levels in lung tissue and expressions of serum inflammatory markers

The results about the MDA, TNF-α, IL-1β, and IL-6 cytokine levels are shown in Table 1. PC induction was found to increase local TNF-α expression in the lungs relative to the control group. Serum IL-1β and IL-6 levels were observed to be significantly increased in the PC group compared to the control group (P=0.002). The PC group exhibited a significantly higher level of MDA, a lipid oxidation marker, than the control group. Hesperetin administration was observed to cause a significant degree of reduction in pulmonary MDA and TNF-α along with serum IL-1β and IL-6 levels in the PC + hesperetin group (P=0.002).

4. Discussion

In the present study, the effects of the natural flavonoid hesperetin on tissue injury and proinflammatory cytokine levels in a rat PC model induced by unilateral blunt chest trauma were investigated using histopathological and

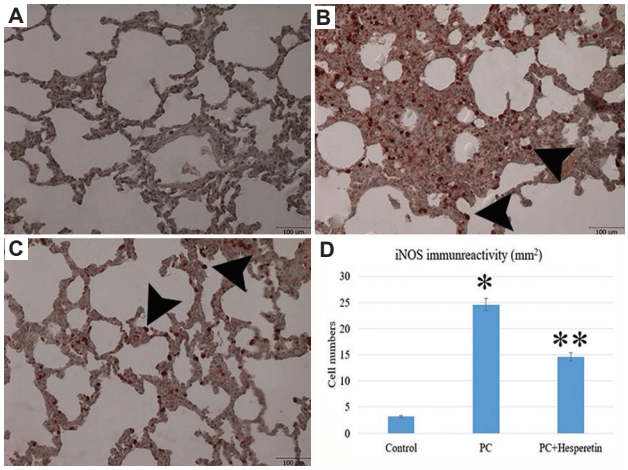

Figure 2. Detection of pulmonary iNOS expression by immunohistochemical means. (A-C) The pulmonary iNOS expression was examined in control (A), PC (B), and PC + hesperetin groups (C). (D) Comparison of iNOS immunoreactivity between groups. For panels (A) to (C): scale bar = 100 μm; arrow denotes iNOS-positive cell; the tissue sections were counterstained with Mayer's hematoxylin; sections were observed microscopically with ×400 magnification (A-C). For panel (D): **P*<0.05 compared to control group; ***P* < 0.05 compared to PC group. Abbreviations: iNOS: Inducible nitric oxide synthase; PC: Pulmonary contusion.

Table 1. Effect of hesperetin on IL-6, IL-1 β, TNF-α, and MDA levels

Group	Lung tissue		Serum	
	TNF-α (pg/mL)	MDA (mmol/mg protein)	IL-6 (pg/mL)	IL-1β (pg/mL)
Control	175.3±9.6	544.7±37.2	45.5±3.9	17.8±3.1
PC	454.3±51.8*	400.8±50.4*	316.2±13.8*	61.9±8.6*
PC+ Hesperetin	297.7±53.7**	479±24.7**	181.8±12.4**	38.3±4.2**

Note: **P*<0.05 compared to control group. **P<0.05 compared to PC group.
Abbreviations: IL: Interleukin; MDA: Malondialdehyde; TNF-α: Tumor necrosis factor-alpha; PC: Pulmonary contusion.

biochemical methods. To the best of our knowledge, this is the first *in vivo* study investigating the effects of hesperetin on the local inflammatory marker (TNF-α), systemic proinflammatory cytokines (IL-1β and IL-6), and lipid oxidation marker (MDA) in a PC model induced by chest trauma. In the current study, oral administration of hesperetin for 7 days significantly ameliorated pulmonary injury in blunt chest trauma-induced PC in rats through strengthening the antioxidant defense system and mitigating inflammation.

Hesperetin, a natural flavonoid abundantly found in fruits of the citrus family such as lemon, grapefruit, and orange, has a variety of pharmacological functions, such as antioxidative, antiapoptotic, and anti-inflammatory effects.[19] Recent research has reported that hesperetin has protective/therapeutic effects on disease models featuring progressive inflammation, especially those of ulcerative colitis, osteoarthritis, acute pulmonary injury, neuronal degeneration, and liver injury.[20-23]

Trauma is the most common cause of death in the first 20 years of life and represents one of the most common causes of fatality after cancer and cardiovascular diseases.[3] Blunt chest trauma damages tissue integrity and may injure tissues and organs in the chest cavity. Vehicular accidents, work accidents, and falls from heights are some examples of the situations where multiple traumas are observed along with blunt chest trauma.[24] The incidence of chest trauma in patients with multiple traumas is 60%, with mortality rates varying from 20% to 25%.[25] Since PC due to blunt chest trauma involves many tissues, organs, and systems, it is thus advisable to adopt a multidisciplinary approach to treating patients with this type of injury.[3] PC developing after blunt chest trauma may cause an exaggerated systemic inflammatory response involving activation of local inflammation mediators, pro-inflammatory cytokines, and complementary systems, which leads to tissue/organ injury.[26,27]

The TNF-α is the chief regulatory cytokine mediating the inflammatory response and acts as a double-edged sword.[28] At optimal levels, TNF-α activation is required for natural immunity, tissue regeneration, and bacterial infections, while excessive expression may trigger apoptosis and cytokine storm.[29] The TNF-α pathway plays a key role in inflammatory diseases, such as acute lung injury (ALI), acute liver injury, and systemic inflammatory response syndrome. Dysregulated TNF-α during acute tissue injury activates neutrophils and macrophages present in tissues and causes release of pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8 from these cells, inducing the inflammatory cytokine cascade. In addition, the increasingly evident polymorphonuclear leukocyte (PML) aggregation in tissues and the heightened apoptosis provoke tissue destruction.[30] A previous study reported increased local TNF-α expression in lung injury due to blunt chest trauma,[31] a finding consistent with the results of the present research that TNF-α expression in lung tissue of the PC group was significantly elevated compared to the control group. In comparison to the control group, the increased TNF-α expression in the PC group aligns well with the histopathological injury score. In addition, according to previous studies, the PC group with blunt chest trauma was found to demonstrate a statistically significant increase in IL-1β and IL-6 levels along with

increased TNF-α expression.[26,32] In the current study, hesperetin attenuated pulmonary TNF-α expression, in addition to obstructing aggregation of inflammatory cells and PML as well as reducing IL-1β and IL-6 levels.

iNOS is one of the enzymes that produce nitric oxide (NO) related to the inflammatory process and have significant biological activity in the lungs.[33] In healthy lung tissue, iNOS is not expressed, but increased iNOS expression in the inflammation and tissue injury processes has been reported in many studies.[34-36] Reaction of NO with superoxide anions, especially reactive oxygen species (ROS), produces peroxynitrite which causes apoptosis and tissue injury in the lungs.[37] In the present study, consistent with previous studies of the PC model,[38,39] rats that had been induced with PC exhibited significantly increased expression of iNOS, as compared to the control group. Previous experimental studies demonstrated that iNOS inhibitors can prevent tissue injury and suppress inflammatory response.[40] In the present study, we detected lower iNOS expression in the PC + hesperetin group, along with reduced histopathological injury score as well as levels of TNF-α, IL-1β, and IL-6, all of which are compatible with results observed in the previous studies. It has been reported that the beneficial effects of hesperetin are attributable to its ability to suppress pro-inflammatory cytokine production and prevent activation of NF-κβ transcription factor, as shown in *in vivo* and *in vitro* experimental studies of ulcerative colitis, hepatitis, LPS-induced ALI, and neuroinflammation.[41-44]

Determining MDA levels, the end product of lipid peroxidation, is one of the most commonly used and reliable methods to assess lipid oxidation in tissue.[45] Compatible with the findings of the present study, high MDA levels in the PC model induced by blunt chest trauma have also been reported elsewhere.[46-48] In this study, we found that hesperetin significantly reduced MDA level in the PC + hesperetin group compared to the PC group. Increasing MDA levels in a doxorubicin-induced oxidative stress model[49] and in a lead acetate-induced oxidative stress model[14] were reported to significantly reduce with administration of hesperetin. Similarly, in an LPS-induced ALI model, hesperetin administration has been found to lower MDA level.[49] These findings obtained in different experimental studies showed that hesperetin administration prevents lipid peroxidation.

Several limitations of this study should be acknowledged. The PC model that was induced using blunt chest trauma may not fully mimic the clinical tableau of the human disease. PC due to blunt chest trauma is generally observed with multiple traumas, while this study created a PC model manifesting only a unilateral trauma. In addition, all animals used in this research were male, so there is no information regarding how females would react to this treatment model. Furthermore, the vital signs and oxygenation status of the animals were not determined during or at the end of the study. This study also did not perform any analysis of bronchoalveolar fluids.

5. Conclusion

The pathological manifestations of unilateral PC induced with blunt chest trauma in rat model include pulmonary edema and hemorrhage, interalveolar septum thickening, and pulmonary inflammation. Besides, PC is associated with increased levels of TNF-α in lung tissues and IL-1 and IL-6 expressions in serum. This study also determined that increased MDA level is a lipid peroxidation marker in PC. To alleviate the PC injury caused by blunt chest trauma, hesperetin stands out as a potential cure by inhibiting the synthesis of TNF-α and iNOS in lung tissues and pro-inflammatory cytokine production in serum, as well as ameliorating tissue damage, pulmonary edema, and inflammation.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Serkan Kaya, İhsan Karaboğa
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Writing – original draft: İhsan Karaboğa
Writing – review & editing: All authors

Ethics approval and consent to participate

All applications in this experimental study were approved by Tekirdag Namık Kemal University Experimental Animals Local Ethics Committee (2018-T115).

Consent for publication

Not applicable.

Availability of data

The data obtained after analysis in the current study can be obtained from the corresponding author on reasonable request.

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ORIGINAL RESEARCH ARTICLE

The *PAI-1 4G/5G* polymorphism, *JAK2V617F* mutation, and their associations with blood cells in Ph-negative myeloproliferative neoplasms

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Abstract

Factors influencing the urokinase-type plasminogen activator system play important roles in pathogenetic processes in Ph-negative myeloproliferative neoplasms (MPNs). In addition, the *JAK2V617F* mutation is a key determinant of outcomes in these diseases. This study evaluated complete blood count (CBC) parameters, the plasminogen activator inhibitor 1 (*PAI-1*) 4G/5G polymorphism, and the *JAK2V617F* mutation in patients with Ph-negative MPNs, aiming to identify possible associations between them. We analyzed results from 56 patients newly diagnosed with Ph-negative MPNs—essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF)—before treatment initiation. The CBC of 475 people from a diagnostic center database served as a population sample for comparison. In patients with Ph-negative MPNs, *PAI-1* genotypes 4G/4G, 4G/5G, and 5G/5G were detected in 11 (19.6%), 29 (51.8%), and 16 (28.6%) cases, respectively. No significant differences in genotype distribution were found among ET, PV, and PMF patients. PMF patients with the 4G/5G genotype had a higher white blood cell (WBC) count compared to those with the 5G/5G genotype ($P = 0.027$). The *JAK2V617F* mutation was found in 44 (78.6%) patients. ET patients with this mutation ($n = 13$) exhibited significantly higher counts of platelets (PLTs), red blood cells (RBCs), and WBCs compared to those without it. The PLT/RBC ratio was significantly higher in all disease categories compared to the population sample, with the highest ratios in ET patients. The PLT/WBC ratio in ET and PV patients was also higher than in the population sample ($P < 0.05$). This relative thrombocytosis is likely clonal in origin, associated with genes responsible for PLT quantitative parameters, JAK-STAT signaling pathway proteins, and factors in the uPA-uPAR-PAI-1/PAI-2 system. These genes share common loci in chromosomes (1p34.1-p34.3, 7q21.1-q21.3, 9p24.1, 19p13.11-p13.2, and 19q13.31-q13.32). Due to their close spatial proximity, these genes can form genetic complexes and mutually influence their expression levels, thereby contributing to the unique pathogenesis of these diseases.

Keywords: Ph-negative myeloproliferative neoplasms; *PAI-1* 4G/5G polymorphism; *JAK2V617F* mutation; Complete blood count

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1. Introduction

Myeloproliferative neoplasms (MPNs) without the presence of the Philadelphia chromosome (Ph-negative), commonly referred to as the "classical" MPNs, include essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). These conditions constitute a distinct subgroup of MPNs. In more than 90% of cases, these conditions are driven by mutations in genes that regulate the expression of proteins of the Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway. The primary driver mutations include *JAK2V617F*, *JAK2* exon 12, myeloproliferative leukemia virus oncogene *(MPL) W515L/K*, and calreticulin (*CALR*).[1,2] These somatic gene mutations impact cytokine signaling, epigenetic regulation, RNA splicing, and signal transduction to transcription factors, leading to the clonal proliferation of pluripotent hematopoietic stem cells and overproduction of cells from various myeloid lineages, which defines the manifestations of Ph-negative MPNs.

The *JAK2V617F* mutation is the most prevalent molecular anomaly, identified in over 95% of patients with PV and 50 – 60% of individuals with ET and PMF.[3,4] Mutations in the *CALR* gene, which result in abnormal interactions with the MPL receptor and subsequent activation and persistent signaling of the JAK2 pathway,[5] are observed in 20 – 30% of patients with ET and PMF.[3,4] Mutations in the *MPL* gene cause constitutive activation of receptors, leading to excessive platelet (PLT) production and other hematopoietic disorders, and have been observed in up to 10% of patients with ET and PMF. Nevertheless, none of the aforementioned mutations are identified in 10 – 25% of patients with ET and PMF.[3,4]

The common features of Ph-negative MPNs include a relatively long and gradual course, development of splenomegaly, fibrous changes in the bone marrow, and clonal tumor evolution. In addition, these conditions display an overproduction of red blood cells (RBCs) and PLTs.[5,6] Despite these characteristics, the mass of tumor stem cells in MPNs is relatively small.

During the course of Ph-negative MPNs, there is a tendency for the development of thromboembolic complications. Thrombosis has been identified as a significant contributor to morbidity and mortality in MPNs, with an estimated prevalence of 20 – 35% in PV patients, 15 – 30% in ET patients, and 10 – 15% in PMF patients.[6] Arterial thrombosis accounts for approximately 60 – 70% of all vascular complications.

The pathogenesis of thrombosis in MPNs is multifactorial, resulting from a complex interaction between blood cells, the endothelium, and the blood coagulation system. An increased number of red blood cells (RBCs), white blood cells (WBCs), and PLTs, in conjunction with qualitative abnormalities, contribute to a prothrombotic phenotype and a hypercoagulable state.[6] This pathogenesis encompasses a range of processes, including aberrant signal transduction, PLT activation, endothelial cell dysfunction, overproduction of tissue factors, formation of PLT-neutrophil aggregates, and hyperviscosity due to an increase in the RBC mass.[7] Different gene polymorphisms contribute to these abnormalities. Mutations in *JAK2*, *CALR*, and *MPL* activate the JAK/STAT pathway, phosphoinositide-3-kinase/Akt (PI3K/Akt) pathway, and mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway. In addition, mutations in genes such as additional sex combs-like 1 (*ASXL1*) and ten-eleven translocation 2 (*TET2*) cause epigenetic changes in DNA and affect the differentiation of hematopoietic cells. These mutations collectively lead to abnormal proliferation rates in blood cells.[7]

The cellular interactions in MPNs create a hyperadhesive and prothrombotic milieu, predisposing patients to thrombosis. PLTs play a pivotal role in these processes, significantly contributing to thromboinflammatory reactions in MPN patients.[8] PLT activation can facilitate the extrinsic coagulation pathway via an endothelial P-selectin-dependent mechanism, enhancing their interaction with WBCs, predominantly neutrophils. PLTs bind to leukocytes through activated endothelial cell surface receptors, GPIbα and PSGL-1, thereby mediating the formation of PLT-leukocyte aggregates.[7]

It is worth noting that RBCs and PLTs constitute the majority of both blood cells and human body cells, at approximately 85% and 4.9%, respectively. This figure does not include their precursors in the bone marrow.[9,10] Consequently, even a minor increase in their absolute number in the blood circulation results in not only hemorheological but also systemic disorders. Unlike leukocytes, which are transient cells using the bloodstream as a communication system to move to various organs and tissues for maturation or function, RBCs and PLTs are stationary blood cells confined to the bloodstream.

The fibrinolytic system plays a key role in thrombolysis and prevention of fibrosis of the intercellular matrix. The urokinase-type plasminogen activator (uPA) system is responsible for the activation and regulation of fibrinolysis. This system is comprised of uPA, the cellular receptor for uPA (uPAR), and its inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2 (uPA-uPAR-PAI-1/PAI-2). The uPA system is involved in a number of processes, including organ stroma remodeling, angiogenesis,

stimulation of several intracellular signaling pathways, apoptosis, inflammation, proliferation, adhesion, cell growth and migration, and oncogenesis.[11] uPA initiates the processes executed by the uPA system by activating plasminogen and converting it to plasmin. In addition to regulating fibrinolysis, plasmin is involved in the activation of matrix metalloproteinases, which can hydrolyze all the main components of the extracellular matrix, thereby playing a key role in the development of invasion, metastasis, cell mobility, and the activation of and release of biologically active regulatory molecules from the extracellular matrix. uPAR, PAI-1, and PAI-2 are responsible for regulating uPA activity.

A comprehensive literature review by Santibanez[12] indicates that the uPA system plays a pivotal role in numerous stages of cancer progression, including extracellular matrix degradation, tumor cell growth and migration, tumor angiogenesis, and epithelial–mesenchymal transition. The overexpression of uPA and uPAR is frequently observed in a multitude of cancer types and is often associated with a poor prognosis. The uPAR receptor serves to localize and enhance the action of uPA and is expressed on the surface of malignant and tumor stromal cells, including fibroblasts.

Degradation and remodeling of surrounding tissues are critical processes in the early stages of tumor progression. These processes contribute to tumor mass expansion, the release of tumor growth factors, cytokine activation, and the induction of tumor cell proliferation, migration, and invasion. The role of proteolytic enzymes of the uPA system in the degradation of the extracellular matrix and tissue remodeling, which are typical of malignant neoplasms, is of pivotal importance.

PAI-1 is also significant in the context of cancer progression.[13] Elevated levels of PAI-1 and soluble uPAR (suPAR) have been observed in late-stage malignancies. Furthermore, the activation of the *PAI-1* gene by the action of the *p53* gene is of great importance for the development of cancer.[14] PAI-1 plays a pivotal role in the pathogenesis of carcinogenesis, angiogenesis, the occurrence of thrombotic complications, and myelofibrosis.

Ph-negative MPNs are characterized by progressive remodeling of the bone marrow stroma, namely the overproduction and deposition of extracellular matrix proteins, neoangiogenesis, and displacement of normal hematopoietic cells by fibrous tissue. SuPAR may serve as a marker of significant tissue remodeling, particularly of the bone marrow in MPNs. In a previous study, patients with MPNs exhibited significantly elevated suPAR levels compared to controls, and suPAR concentration was significantly correlated with the tissue plasminogen activator (tPA):PAI-1 complex. Consequently, elevated plasma levels of suPAR in patients with MPNs may indicate an increase in uPAR production in the bone marrow, resulting in enhanced bone marrow remodeling.[15]

PAI-1 is not only a significant inhibitor of plasmin but also an important regulator of the uPA fibrinolytic system. PAI-1 is subject to modulation by a multitude of regulatory factors, including hormones, cytokines, and growth factors. Among these, transforming growth factor-β (TGF-β) acts as a potent inducer of PAI-1 expression.[16] PAI-1 represents a pivotal downstream target of the TGF-β pathway, which is the primary driver of the fibrotic response. TGF-β positively regulates PAI-1 gene expression via two main pathways, including Smad-mediated canonical and non-canonical pathways. The overexpression of PAI-1 has been demonstrated to reduce the degradation of the extracellular matrix by perturbating the plasminogen activation system. Elevated PAI-1 levels inhibit the proteolytic activity of tissue plasminogen activator and uPA, which could contribute to excessive matrix deposition.[17]

PAI-1 is involved in the processes of invasion and metastasis by inhibiting uPA and the proteolytic cascade, which includes the activation of plasminogen and matrix metalloproteinases. However, PAI-1 can also inhibit tumor-destroying proteinases and protect the tumor from proteolysis. Consequently, a high level of *PAI-1* expression may serve as a predictor of an unfavorable prognosis and indicate an increased risk of metastasis and tumor recurrence. In contrast to *PAI-1*, high expression of *PAI-2* is associated with an increase in life expectancy, a decrease in the number of metastases, and a decrease in the rate of tumor growth in various types of cancer. It is important to note that the uPA system is regarded as a promising target for anticancer therapy.

Elevated levels of PAI-1 may contribute to the formation of cancerous cells, possibly due to the fact that the reactions caused by PAI-1 can result in mutations in genes that regulate cell growth and division. Furthermore, PAI-1 has been demonstrated to stimulate inflammation, which in turn can contribute to the development of cancer.

PAI-1 is a direct target for the microRNA miR-145-5p, which is a direct target of NKX2-1 antisense RNA 1 (*NKX2-1-AS1*). Its overexpression has been demonstrated to promote cell proliferation, metastasis, invasion, and angiogenesis. Furthermore, the NKX2-1-AS1/miR-145-5p axis induces the translation of PAI-1, thereby activating the vascular endothelial growth factor receptor 2 (VEGFR-2) signaling pathway, which promotes tumor progression and angiogenesis.[18]

The insertion or deletion of guanosine at 675 bp upstream of the transcription start site results in the presence of a 4G/5G gene polymorphism in the promoter of the *PAI-1* gene. This polymorphism is characterized by three distinct genotypes: 4G/4G, 4G/5G, and 5G/5G, which regulate the expression level of *PAI-1*. The plasma PAI-1 level is the highest in genotype 4G/4G, followed by genotype 4G/5G, and is the lowest in genotype 5G/5G. It has been suggested that the 4G/5G gene polymorphism and the level of PAI-1 protein are closely related to arterial and venous thrombosis.[19] The data presented by Zhang *et al.*[20] indicate that the incidence of thrombotic events in patients with ET is significantly higher in those with the 4G/4G polymorphism of the *PAI-1* gene than in those with the 4G/5G or 5G/5G genotypes. Furthermore, the 4G/4G polymorphism of the *PAI-1* gene and infection are independent risk factors for thrombotic events in patients with Ph-negative MPNs. Finally, the data demonstrate that the *PAI-1* 4G/4G polymorphism and infection in ET and PV patients with the *JAK2V617F* mutation are high-risk factors for thrombotic events. In patients with ET and PV with prothrombotic coagulation disorders, the level of PAI-1 is significantly increased.[21]

Therefore, elucidating the role of PAI-1 and its gene polymorphism, as well as *JAK2* mutations in the pathogenesis of Ph-negative MPNs, can be of great theoretical and practical importance.

This study aimed to evaluate various parameters of complete blood count (CBC), including several integral cell indices (ICIs), the −675 4G/5G polymorphism of the *PAI-1* gene, and the presence of the *JAK2V617F* mutation. Furthermore, the study sought to identify potential associations among these parameters in patients newly diagnosed with Ph-negative MPNs, while considering the involvement of the uPA/uPAR system in the pathogenesis (Table 1).

2. Methods

2.1. Subject data and control sample

This study examined the blood samples from 56 patients (34 women and 22 men) diagnosed with ET (n = 22), PV (n = 19), and the pre-fibrotic phase of PMF (n = 15) before the initiation of treatment. The age of patients ranged from 19 to 82 years (median: 53 years). The examinations were conducted at the Laboratory of Molecular Genetics, Institute of Blood Pathology and Transfusion Medicine, National Academy of Medical Sciences (NAMS) of Ukraine, during 2021 – 2022. The diagnosis of Ph-negative MPNs was established on the basis of a comprehensive evaluation of clinical, hematological, and molecular genetic examinations. The 2016 World Health Organization criteria[22] were used for diagnosis, incorporating the detection of *JAK2V617F* and CALR mutations, including the two most frequent CALR mutations, a 52 base pair (bp) deletion and a 5bp insertion. At the time of diagnosis, deep vein thrombosis in the lower leg or thigh was present in three (5.4%) patients with Ph-negative MPNs, among whom one had PMF and two had ET.

As the control group, we used data from a population sample in the database of the Medis Laboratory and Diagnostic Center, Lviv, Ukraine, in 2020, comprising CBC of 475 individuals (245 women and 230 men).

2.2. Presence of the *JAK2V617F* mutation

DNA isolated from patients' blood cells was used for detecting the *JAK2V617F* mutation. Venous blood sampling was carried out using vacuum tubes with K2 EDTA with a capacity of 3 mL. DNA isolation was carried out with Chelex 100 Resin (Bio-Rad, USA). The obtained DNA was frozen in a freezer at −20°C for long-term storage. Detection of the *JAK2V617F* mutation was conducted using the real-time polymerase chain reaction

Table 1. The impact of PAI-1 overexpression caused by excessive PLT production/activation on the pathogenesis of Ph-negative MPNs

Factors contributing to *PAI-1* overexpression	Consequent pathogenesis		
	Blood	ECM	Cells
(i) Increased PLT count (ii) Activated PLT (iii) The presence of *PAI-1* 4G/5G polymorphism	Increased formation of the PAI-1–tPA complex inhibits Pg activation, thereby reducing the conversion of Pg to Pm and ultimately heightening the risk of thrombosis	(i) Increased formation of the PAI-1–uPA complex inhibits Pg activation, thereby reducing the conversion of Pg to Pm, ultimately heightening the risk of myelofibrosis (ii) Increased formation of the PAI-1–uPA complex inhibits MMP activation, ultimately heightening the risk of myelofibrosis	Increased formation of the PAI-1–uPA–uPAR (Ly6/uPAR family members) complex alters the activation of signaling pathways and transcriptional regulation, heightening the risk of angiogenesis and tumor progression

Abbreviations: ECM: Extracellular matrix; Ly6: Lymphocyte antigen 6; MMP: Matrix metalloproteases; MNP: Myeloproliferative neoplasm; PAI-1: Plasminogen activator inhibitor 1; Pg: Plasminogen; PLT: Platelet; Pm: Plasmin; tPA: Tissue plasminogen activator; uPA: Urokinase-type plasminogen activator; uPAR: Urokinase-type plasminogen activator receptor.

(PCR) method by setting up a multiplex reaction. Sets of reagents were used to detect polymorphisms in the human genome through the single nucleotide method. Allele1 (wild type) was labeled with the fluorophore HEX, while Allele2 (mutated) was determined using the FAM channel. Amplification was performed on the CFX96™ Real-Time System (Bio-Rad, USA). The results were analyzed using CFX Maestro™ Software (Bio-Rad, USA). The amplification reaction was performed according to the following protocol: one cycle of temperature 80°C for 2 min; one cycle of temperature 94°C for 3 min; 40 cycles of temperature 94°C for 15 s; and 40 cycles of temperature 64°C for 40 s (reading phase).

2.3. Distribution of genotypes of the polymorphic locus 4G/5G of the *PAI-1* gene

The distribution of genotypes and alleles of the polymorphic locus –675 4G/5G ins/del of the *PAI-1* gene was determined through molecular genetic analysis of the DNA sequence using the PCR method in high-resolution melting mode. This was accomplished using the GenETics Haemostatic Disease (PAI-1/ITGB3) Kit from Adaltis (Italy) on the CFX96TMReal-Time System (Bio-Rad, USA). The results were analyzed using the CFXMaestro™ Software and RealBest Diagnostics programs.

2.4. Blood cell count and ICIs

Hematopoiesis features were assessed through automated counting of RBCs, PLTs, and WBCs. ICIs were calculated, including the PLT/WBC ratio and C×PLT/RBC. The conventional coefficient, C=20, was used to estimate the synchronization of reproduction of the PLT and RBC populations, considering that the PLT population typically recovers 20 times faster than the RBC population. Therefore, approximately the same amount of PLTs and RBCs is produced per conventional unit of time.

2.5. Statistical analysis

Statistical processing of the obtained data was performed using the Statistica for Windows software package (Statsoft, USA). Quantitative parameters were compared using the Mann–Whitney *U*-test and were presented as median (interquartile range). The Fisher's test and the χ^2 test were used to compare qualitative parameters.

3. Results

3.1. Blood cell count and ICIs

Before the initiation of treatment, thrombocytopoiesis was predominant over erythropoiesis and leukopoiesis in all patients with Ph-negative MPNs. The C×PLT/RBC index was significantly higher in patients with ET (3.70 [2.84 – 4.01]), PV (1.45 [0.85 – 2.12]), and PMF (1.79 [1.10 – 2.52]) compared to the control group (0.96 [0.79 – 1.16]) ($P < 0.0001$, $P = 0.012$, and $P < 0.0001$, respectively). Similarly, the PLT/WBC ratio was significantly elevated in patients with ET (83.8 [71.7 – 124.1]) and PV (43.7 [34.0 – 69.3]) compared to the control group (36.6 [28.3 – 45.2]), with $P < 0.0001$ and $P = 0.031$, respectively (Figure 1).

3.2. *JAK2V617F* mutation and its association with the blood cell count and ICIs

The *JAK2V617F* mutation was detected in 44 (78.6%) patients: 13 (59.1%) with ET, 18 (94.7%) with PV ($P = 0.011$ compared to the ET group), and 13 (86.7%) with PMF. No significant differences in ICIs were observed between subgroups of patients with and without the *JAK2V617F* mutation. However, patients with ET who had this mutation showed significantly higher blood cell counts compared to those without the mutation: PLT counts were 918 (837 – 1025) $\times 10^9$/L vs. 732 (583 –837) $\times 10^9$/L ($P = 0.021$), RBC counts were 4.96 (4.44 –5.35) $\times 10^{12}$/L vs. 4.19 (4.05 –4.54) $\times 10^{12}$/L ($P = 0.015$), and WBC counts were 11.5 (7.1 – 15.1) $\times 10^9$/L vs. 6.3 (5.9 – 7.5) $\times 10^9$/L ($P = 0.012$). This finding suggests that in patients with ET and the *JAK2V617F* mutation, the increase in blood cell counts is likely due to excessive production resulting from the activation of the JAK-STAT pathway in hematopoietic stem cells and progenitor cells.[23]

3.3. The *PAI-1* 4G/5G genotypes and their associations with the *JAK2V617F* mutation

In patients with Ph-negative MPNs, the *PAI-1* 4G/4G, 4G/5G, and 5G/5G genotypes were detected in 11 (19.6%), 29 (51.8%), and 16 (28.6%) cases, respectively. The distribution rate was 4G/5G > 5G/5G > 4G/4G. According to molecular genetic analysis conducted by Makukh *et al.*,[24] the distribution of these genotypes in DNA samples of 225 healthy residents of the Western Ukrainian region was 33.33% for 4G/4G, 45.33% for 4G/5G, and 21.33% for 5G/5G, with the distribution rate being 4G/5G > 4G/4G > 5G/5G. The frequency of the 4G allele was 45.5% in the examined MPN patients and 56.0% in the general population. No significant differences in the distribution of *PAI-1* gene genotypes and alleles were found between the general population sample and our study.

In patients with ET, the detection rates of the genotypes 4G/4G, 4G/5G, and 5G/5G were in the following order: 5G/5G > 4G/5G > 4G/4G (40.9%, 36.4%, and 22.7%, respectively). In patients with PV, the order was 4G/5G > 5G/5G > 4G/4G (63.15%, 21.05%, and 15.8%, respectively). For patients with PMF, the order was 4G/5G > 5G/5G = 4G/4G (60.0%, 20.0%, and 20.0%, respectively).

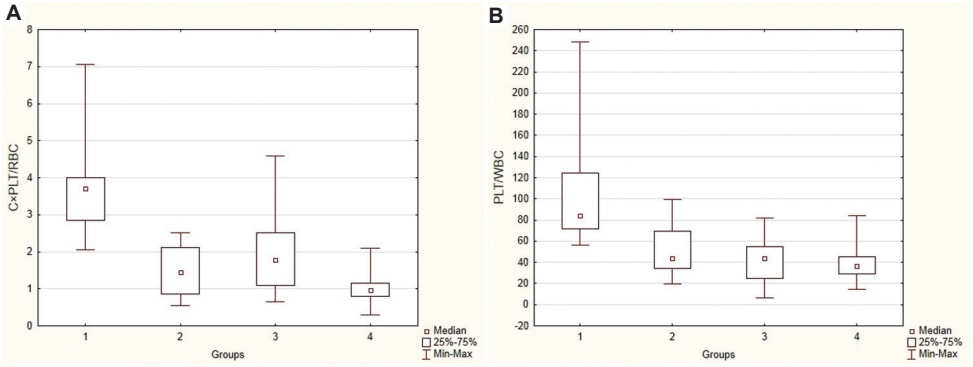

Figure 1. The (A) C×PLT/RBC index and (B) PLT/WBC ratios in patients with essential thrombocythemia (Group 1), polycythemia vera (Group 2), and primary myelofibrosis (Group 3). Group 4 represents the control
Abbreviations: PLT: Platelet; RBC: Red blood cell; WBC: White blood cell.

The frequency of the 4G allele was 40.9% in ET patients, 47.4% in PV patients, and 50.0% in PMF patients. There were no significant differences in the detection rates of 4G/4G, 4G/5G, and 5G/5G genotypes or the 4G allele among the groups of patients with ET, PV, and PMF. In addition, no significant differences were found in the distribution of alleles and genotypes of the 4G/5G polymorphic locus of the *PAI-1* gene in patients with ET, PV, and PMF compared to the general population sample.[24]

Three patients with Ph-negative MPNs and thrombotic complications at the time of diagnosis had *PAI-1* gene 4G/4G or 4G/5G polymorphisms, along with mutations in the prothrombin (*G20210A*), FV Leiden (*G1691A*), or folate cycle enzyme genes, and were associated with hyperhomocysteinemia.

The molecular genetic analysis of the polymorphic locus −675 4G/5G of the *PAI-1* gene in 44 patients with MPNs and the presence of the *JAK2V617F* mutation revealed the 4G/4G, 4G/5G, and 5G/5G genotypes of the *PAI-1* gene in ten (22.7 %), twenty two (50.0%), and twelve (27.3%) cases, respectively. The detection rate was 4G/5G > 5G/5G > 4G/4G. In 12 patients without *JAK2* mutations, 4G/4G, 4G/5G, and 5G/5G genotypes occurred in one (8.3%), seven (59.3%), and four (33.3%) cases, respectively ($P > 0.05$ compared to carriers of the *JAKV617F* mutation). The detection rate was 4G/5G > 5G/5G > 4G/4G. The 4G allele was detected in 47.7% of patients with the *JAK2* mutation and in 37.5% of patients without the *JAK*2 mutation ($P > 0.05$).

3.4. Associations of the *PAI-1* 4G/5G polymorphism with blood cell count and ICIs

Regarding the potential association of the −675 4G/5G polymorphism in the *PAI-1* gene with the cellular composition of blood in patients with newly diagnosed Ph-negative MPNs before the initiation of treatment, significant differences were observed. In patients with PMF, those with the 4G/5G genotype had significantly higher WBC counts compared to patients with the 5G/5G genotype, specifically 9.60 (8.49 – 11.19) × 10^9/L vs. 7.31 (5.10 – 8.30) × 10^9/L ($P = 0.027$). In addition, in patients with the 4G/4G genotype, there was a tendency toward higher WBC counts compared to patients with the 5G/5G genotype, 10.04 (9.70 – 11.70) × 10^9/L vs. 7.31 (5.10 – 8.30) × 10^9/L ($P = 0.081$). In patients with ET, those with the 4G/4G genotype demonstrated a tendency toward a lower C×PLT/RBC index compared to patients with the 5G/5G genotype, specifically 2.53 (2.46 – 3.78) vs. 3.81 (3.70 – 4.27) ($P = 0.083$) (Table 2).

In patients with ET, both ICIs were significantly higher across subgroups with different genotypes compared to the control group. This finding reflects a predominance of thrombocytopoiesis over erythropoiesis and leukopoiesis, regardless of −675 4G/5G polymorphism in the *PAI-1* gene. Similarly, in patients with PV with 4G/4G and 5G/5G genotypes and in patients with PMF with 4G/4G and 4G/5G genotypes, the C×PLT/RBC index was also higher compared to the control group.

4. Discussion

In carcinogenesis, disorders in the multifunctional uPA-uPAR-PAI-1/PAI-2 system, and particularly an increase in the level of the main uPA inhibitor, PAI-1, are of great importance.[13,25] A significant rise in PAI-1 levels was also found in patients with Ph-negative MPNs.[21] PAI-1 levels may depend on both the genotype of the −675 4G/5G polymorphic locus of its gene[26] and the number of PLT, which are the main source of PAI-1.

The results of this study reveal that in patients with newly diagnosed Ph-negative MPNs, there were no significant differences in the distribution of alleles and genotypes of the −675 4G/5G polymorphic locus of the *PAI-1* gene, either between groups of patients with ET, PV, and PMF,

Table 2. Integral cell indices in patients with Ph-negative myeloproliferative neoplasms depending on the genotype of the polymorphic locus –675 4G/5G of the *PAI-1* gene

PAI-1 4G/5G genotype	ET		PV		PMF		Control	
	C×PLT/RBC	PLT/WBC	C×PLT/RBC	PLT/WBC	C×PLT/RBC	PLT/WBC	C×PLT/RBC	PLT/WBC
4G/4G	2.53 (2.46 – 3.78)*	73.7 (71.7 – 81.9)*	1.57 (1.11 – 1.64)*	43.7 (34.0 – 62.4)	2.52 (1.06 – 4.58)*	62.8 (24.4 – 81.65)	0.96 (0.79 – 1.16)	38.2 (29.3 – 45.2)
4G/5G	3.67 (2.96 – 4.20)*	98.0 (75.0 – 133.1)*	1.24 (0.82 – 1.67)	39.4 (33.3 – 57.6)	1.84 (1.45 – 2.36)*	40.2 (30.0 46.25)		
5G/5G	3.81 (3.7 – 4.27)*	101.0 (80.0 – 124.1)*	2.23 (1.48 – 2.43)*	74.5 (44.3 – 89.6)	1.37 (0.64 – 1.79)	45.0 (19.6 – 47.2)		

*$P<0.05$ compared with the control group.

Abbreviations: ET: Essential thrombocythemia; PLT: Platelet; PMF: Primary myelofibrosis; PV: Polycythemia vera; RBC: Red blood cell; WBC: White blood cell.

or in comparison with the general population sample. In addition, no significant associations were observed between the distribution of *PAI-1* gene polymorphism genotypes and the presence of the *JAK2V617F* mutation. This finding is consistent with the observation of Zhang *et al.*[20] in patients with PV and ET, namely that the incidence rate of PAI-1 genotype in JAK2V617F mutation-positive patients was 4G/5G (37.5%) = 5G/5G (37.5%) > 4G/4G (25%) and 4G/4G (48.2%) > 4G/5G (33.3%) > 5G/5G (18.5%), respectively. Compared with *JAK2V617F* mutation-negative patients, the difference was not statistically significant.

In examined patients with PMF, the 4G/5G heterozygous genotype of the *PAI-1* gene was associated with a higher WBC count. It is well established that leukocytosis can predict the occurrence of thrombotic events in myelofibrosis.[27] The association of a higher WBC count with the thrombophilic *PAI-1* 4G/5G polymorphism, as observed in our study, may be crucial in determining the elevated thrombosis risk. Furthermore, data presented by Ohyashiki *et al.*[28] indicated that leukocytosis is associated with thrombosis also in patients with ET.

A significant change in the balance of blood cells, particularly an increase in PLT count, was found in the examined patients. A relative increase in PLT count compared to WBC count was observed in patients with ET and PV. In addition, a relative increase in PLT count compared to RBC count was observed in patients with ET, PV, and PMF. This phenomenon was associated with specific genotypes of the *PAI-1* gene, namely 4G/4G and 5G/5G in patients with PV, and 4G/4G and 4G/5G in patients with PMF. The relative thrombocytosis observed in our study is most likely of clonal origin, as evidenced by the lack of correlation between thrombopoietin levels and PLT count in patients with PV and ET.[29] The available literature indicates that an increase in the number of PLTs may result in elevated plasma levels of PAI-1 in patients with Ph-negative MPNs.[21]

PLTs are acknowledged as the primary cell regulating hemostasis. Their vascular importance is attributed to their essential role in thrombosis. PLTs mediate complex vascular homeostasis via specific receptors and granule release, RNA transfer, and mitochondrial secretion.[30]

The existing literature on the role of increased PLT count in the pathogenesis of thrombosis in MPNs appears to be controversial. Nevertheless, it does not negate the significance of several other evidence-based indications that PLTs may contribute to thrombotic risk. For example, elevated neutrophil-PLT aggregation, accompanied by augmented expression of activation markers CD11b and CD62P, has been observed in individuals with ET and PV. This phenomenon may be linked to their history of

thrombotic complications.[31] It can be speculated that the higher counts of PLTs and WBCs observed in *JAK2V617F* mutation-positive patients with ET in our study may result in such neutrophil-PLT aggregation.

PLTs retain high levels of active PAI-1. The concentration of PAI-1 in plasma has been found to correlate with the number of PLT in the blood.[32] In fact, more than 50% of PLT PAI-1 is in the active form.[33] Since PLTs retain mRNA from megakaryocytes and have the ability to synthesize certain proteins, they can synthesize PAI-1 *de novo*. *In vitro* studies have demonstrated that the amount of PAI-1 synthesized in 24 h is 35 times higher than that required to maintain normal plasma levels.[32]

It is more common for patients with clonal thrombocytosis to experience thrombosis than for those with secondary thrombocytosis. In cases of clonal thrombocythemia, plasma levels of PAI-1 antigen and activity are significantly higher than in the reactive thrombocytosis group. Given that PLTs continuously produce substantial quantities of active PAI-1 and represent the primary reservoir of PAI-1 in plasma, the thrombotic tendency observed in MPNs may be associated with an elevation in PAI-1 levels.[34] A significant increase in PLT PAI-1 levels was observed in patients with ET with thrombotic complications compared with patients with ET without thrombotic complications.[35]

PLT PAI-1 plays a pivotal role in the development of arterial thrombosis. Arterial thrombi containing approximately two to three times more PAI-1 than venous thrombi are more commonly detected in patients with MPNs.[36] In an animal experiment, it was demonstrated that PLT-derived PAI-1 provides a high concentration of PAI-1 in arterial thrombi.[37,38]

The quantitative parameters of PLTs, such as number, volume, function, and kinetics (production and survival), are genetically determined and depend on the expression level of many genes.[39] Several genes regulating PLT quantitative parameters and those governing the expression of proteins of the JAK-STAT signaling pathway and the uPA-uPAR-PAI-1/PAI-2 system are located on adjacent chromosomes. In particular, the *MPL* gene (1p34.2) is linked to *CSF3R* (1p34.3) and *JAK1 (*1p31.3) genes. The *JAK2* gene (9p24.1) is not only located next to the PLT-associated genes, such as *AK3 (*9p24.1) and *RCL1 (*9p24.1), but also next to the *PGR* gene (9p24.1). The *TPM4* gene (19p13.12) is linked to *CALR* (19p13.13), *JAK-3 (*19p13.11), and *EPOR* (19p13.2) genes. It should be noted that one of the PLT-associated genes, namely the *FLJ36031-PIK3CG* (7q22.3) gene, is located next to the *SERPINE1* gene, which encodes PAI-1, as well as to the *EPO* gene (7q22.1).[40] This region of chromosome 7 is important in the regulation of hematopoiesis, and the detection of genetic abnormalities in it is associated with the development of myeloid neoplasms.[41,42] The *EXOC3L2* gene (19q13.32), which belongs to the regulators of PLT quantitative parameters, is located next to the cluster of a large family of genes *PRV* (CD177) and *Ly6/uPAR* (19q13.31), which form one functional block, whose overexpression is typical for PV and ET. The significance of uPAR in maintaining normal PLT survival is noteworthy.[43]

Thus, the genes responsible for the expression of uPA-uPAR-PAI-1/PAI-2, PLT quantitative parameters, and JAK-STAT signaling pathway proteins share several common chromosomal regions (1p34.1-p34.3, 7q21.1-q21.3, 9p24.1, 19p13.11-p13.2, and 19q13.31-q13.32). Due to this close spatial location, these genes can form specific genetic complexes and mutually influence their expression levels through genetic regulation and gene interactions. Given that phenotypic features of tumors are determined by many genes, a comprehensive study of mutations and gene dysregulation in the above-mentioned chromosomal regions will not only contribute to a better understanding of the molecular genetic nature of Ph-negative MPNs but also aid in developing new approaches to personalize the treatment of patients with these diseases.

The *JAK2* gene, located on chromosome 9p24.1, encodes a non-receptor tyrosine kinase involved in cell growth, differentiation, development, and histone modification. Ligand binding to type I or II receptors associated with the JAK2 protein prompts JAK2 to phosphorylate tyrosine within the cytokine receptor's cytoplasmic region, thereby generating several docking sites for STAT protein recruitment and phosphorylation. Phosphorylated STAT proteins dimerize in the cytoplasm and translocate into the nucleus to further activate genes.[7]

One of the most prevalent mutations in the *JAK2* gene is the *V617F*, a somatic gain-of-function mutation altering the 1849th coding nucleotide from guanine to thymine, resulting in the replacement of valine with phenylalanine. The *JAK2V617F* mutation serves as a molecular marker for MPNs.[7]

This study demonstrates that the *JAK2V617F* mutation was identified in 78.6% of patients diagnosed with MPNs. This mutation was observed in 59.1% of patients with ET, 94.7% of patients with PV, and 86.7% of patients with PMF. This detection rate is consistent with literature data, indicating that the frequency of the *JAK2V617F* mutation in patients with ET, PV, and PMF ranges from 31.3 to 72.1%, 46.7 to 100%, and 25.0 to 85.7%, respectively.[27,44]

A body of evidence indicates that the *V617F* mutation increases the risk of thrombosis and may serve as a predictive

biomarker of thrombotic events in MPNs. Thrombosis is significantly and frequently observed in patients with the *V617F* mutation compared to those without. Patients with *JAK2V617F* mutation-positive MPNs exhibit high levels of hemoglobin and hematocrit, along with low levels of erythropoietin, an increase in WBC count, and a higher frequency of thrombosis.[7,28] In addition, our study found that the *V617F* mutation in patients with ET is associated with higher levels of WBCs, RBCs, and PLTs.

It has been demonstrated that alterations in the gene expression profile of megakaryocytes can lead to circulating PLTs with altered hemostatic or inflammatory function. PLT activation can be caused by abnormalities in hematopoietic stem cells due to dysfunction associated with driver mutations that lead to hyperactive JAK2-dependent signaling.[45] Furthermore, it was demonstrated that several genes involved in thrombin signaling and PLT activation were downregulated in *CALR*-mutated patients in comparison to *JAK2V617F*-mutated ones. This observation correlates with a lower thrombotic susceptibility in the former.[46]

Furthermore, PLT activation can be initiated by the overproduction of PLT and direct interaction with activated leukocytes, endothelial cells, and various mediators. Consequently, the PLT transcriptome integrates specific information obtained from both clonal cells and megakaryocytes that are not involved in the malignant clone, as well as from the bone marrow microenvironment and blood circulation, which largely determines their activity. Consequently, transcriptome analysis and the detection of changes in gene expression at a specific time point will facilitate a more profound comprehension of the underlying mechanisms that contribute to PLT dysfunction in MPNs.[47]

The present study demonstrates that to assess the balance and kinetics of blood cells in patients with Ph-negative MPNs and to choose optimal clinical decisions, it is advisable to determine the ratio between different populations of blood cells along with the analysis of quantitative parameters of the CBC. Using the ratio between PLT and other blood cells in our study, we found a relative increase in the PLT count compared to the RBC count in patients with Ph-negative MPNs, not only with ET but also with PV and PMF. Certain relationships were identified between the count of peripheral blood cells and mutations in the *PAI-1* and *JAK2* genes in these patients. Further studies on a larger sample of patients using multivariate clinical and molecular genetic analysis are needed to study the influence of the 4G/5G polymorphism of the *PAI-1* gene on the development of myelofibrosis and prothrombotic disorders of hemostasis, as well as to study the importance of PLT, as the main source of PAI-1, in the course of Ph-negative MPNs.

5. Conclusion

The *JAK2V617F* mutation was detected in 78.6% of patients with newly diagnosed Ph-negative MPNs. In patients with ET, it is associated with a higher count of all blood cells.

The 4G/4G, 4G/5G, and 5G/5G genotypes of the *PAI-1* gene were detected in patients with MPNs in 19.6%, 51.8%, and 28.6% of cases, respectively, which is not significantly different from their distribution in the general population and does not depend on the presence of a mutation in the *JAK2* gene.

A relative increase in PLT count compared to RBC count and WBC count was observed in patients with MPNs. The heightened ratio of PLTs over RBCs is associated with −675 4G/5G polymorphism in the *PAI-1* gene in patients with PV and PMF. In addition, in patients with PMF, the 4G/5G heterozygous genotype of the *PAI-1* gene is associated with a higher WBC count.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Yevhen Dzis
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Writing – review & editing: Yevhen Dzis, Oleksandra Tomashevska

Ethics approval and consent to participate

Ethical approval was sought and obtained in accordance with the World Medical Association (WMA) Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects, as well as the directives outlined in the Ministry of Health of Ukraine No. 690 dated September 23, 2009, No. 944 dated December 14, 2009, and No. 616 dated August 3, 2012.

Consent for publication

Not applicable.

Availability of data

Data used in this work are available from the corresponding author upon reasonable request.

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MINI-REVIEW

Folic acid supplementation for stroke prevention: The devil is in the details

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Abstract

Cerebrovascular disease (CVD) is the second leading cause of death worldwide. In addition to the traditional risk factors such as hypertension, diabetes, and hyperlipidemia, nutritional folate deficiency may be an important risk factor for CVD, especially in low-income countries. Folic acid supplementation has been considered for stroke prevention, but trial results have been variable. Therefore, in general, stroke patients do not receive folic acid supplementation routinely, partly due to the lack of consensus regarding such necessity. To be metabolically active, the synthetic folic acid, which is often taken as a supplement, needs to be enzymatically converted to 5-methyltetrahydrofolate (5-MTHF) by the endogenous enzyme methylenetetrahydrofolate reductase (MTHFR). 5-MTHF promotes homocysteine catabolism while improving endothelial function and reducing superoxide generation. It has been shown that supplementation with synthetic folic acid reduced the incidence of ischemic strokes in individuals with hypertension, but the efficacy of folic acid supplementation in primary prevention of ischemic strokes was markedly reduced in the subset of hypertensive patients with mutations in the *MTHFR* gene. Furthermore, supplementation with synthetic folic acid may promote accumulation of unmetabolized free folic acid which may increase risk of cancer, immune suppression, and cognitive impairment, especially in patients with mutations in *MTHFR*. Since *MTHFR* genotyping is neither feasible nor cost-effective in the vast population of patients at risk of ischemic stroke, supplementation with low-dose 5-MTHF merits examination in large well-designed clinical trials.

Keywords: Folic acid; Folate; Methylenetetrahydrofolate reductase; 5-methyltetrahydrofolate; Stroke; Cerebrovascular disease; Hypertension; Cardiovascular disease

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1. Folic acid deficiency and cerebrovascular disease (CVD)

CVD is the leading cause of long-term disability and the second leading cause of death worldwide.[1] Approximately 795,000 new or recurrent acute strokes secondary to CVD

occur annually in the United States (US), and mortality from CVD is increasing significantly among younger adults.[1] Deaths and disability due to strokes are expected to rise alarmingly over the next 30 years, with an annual stroke mortality projected to increase by 50% or 3 million by 2050 according to a new report from the World Stroke Organization – *Lancet Neurology* Commission.[2] Nearly 85% of strokes are ischemic.[3] High morbidity, impact on quality of life, and mortality from ischemic stroke have underlined the importance of preventing this dreaded complication in patients at risk.

Patients with hypertension are three to four times more likely to suffer a stroke compared to non-hypertensive patients.[4] Hypertension remains the leading cause of ischemic stroke,[5] with 54% of strokes attributable to hypertension. Hypertension is the most common modifiable risk factor for ischemic stroke globally,[6] followed by diabetes, smoking, obesity, atrial fibrillation, and illicit drug use. Hypertension increases the susceptibility to ischemic stroke by promoting and accelerating atherosclerotic plaque formation, vascular smooth muscle cell hypertrophy, and remodeling of systemic and cerebral arteries, leading to arterial occlusion and ischemic injury.[6]

Strikingly, 86% of global deaths and 89% of global disability-adjusted life-years lost due to stroke in 2020 occurred in low- and middle-income countries (LMICs) and continue to grow faster in LMICs than in high-income countries.[2,7] The factors underlying the differences in incidence of stroke in low versus high-income countries are poorly understood although a good understanding of these factors is essential to tackle this epidemic. An obvious factor is the relatively poor availability of health-care services including medications, which leads to poor control of hypertension in LMICs.[8] Another important factor to consider is folate deficiency, given the important role of folate in vascular health. The biologically active form of folate, 5-methyltetrahydrofolate (5-MTHF) is essential for homocysteine catabolism.[9] In addition, 5-MTHF directly improves endothelial nitric oxide synthase coupling, thereby improving endothelial function while reducing vascular superoxide production.[10,11]

Folate is not synthesized in the body and must be obtained through the diet.[12] Due to difficulty in achieving adequate dietary intake of naturally occurring folates in the general population, folic acid fortification of food and supplementation has become common practices.[12] In the US and Canada, folic acid fortification of enriched grain products was fully implemented by 1998.[13] The slow decline in stroke mortality observed between 1990 and 1997 accelerated markedly in 1998 to 2002 in nearly all population strata.[13] The decline in stroke mortality was statistically significant and could not be accounted for by any changes in other recognized risk factors.[13] During the same time period, in the absence of folic acid fortification, there was no decline in stroke mortality in the United Kingdom.[13] These findings suggest that folic acid fortification may help to reduce stroke-related mortality.

2. Folic acid versus 5-MTHF for prevention of ischemic stroke

Patients with cardiovascular disease (CVD) are at increased risk of stroke.[13] Tian *et al.*[14] performed a meta-analysis of 65,790 patients with CVD and observed a significant reduction in stroke risk after folic acid supplementation (RR = 0.90; 95% CI: 0.84 – 0.97; P = 0.05). Further stratified analysis revealed greater beneficial effects in populations from regions with no or partly fortified grain. The authors concluded that in patients with CVD, folic acid supplementation reduces stroke risk, especially in patients consuming grain that is either not fortified or only partly fortified. This is consistent with randomized controlled trials demonstrating lower risk of future stroke in patients receiving folic acid supplementation in countries without mandatory folic acid food fortification.[15] The control group and the experimental groups were balanced in the distribution of patients with comorbid conditions including acute myocardial infarction with hypertension, hyperlipidemia, coronary artery disease, and end-stage renal disease.[15]

Folic acid supplementation has been recommended for primary stroke prevention in patients with hypertension.[16] In a meta-analysis of patients with CVD, folic acid supplementation reduced risk of stroke and overall CVD by 10% and 4%, respectively.[16] Although epidemiologic studies have demonstrated lower serum homocysteine concentrations associated with reduced risk of stroke, randomized controlled trials of folic acid to reduce homocysteine levels have yielded mixed results regarding stroke prevention. Lee *et al.*[17] performed a meta-analysis of 13 randomized controlled trials that had enrolled about 39,000 participants and observed a trend toward a benefit with folic acid supplementation, which was not statistically significant. The authors concluded that folic acid supplementation did not demonstrate a major effect in averting stroke. In stratified analyses, a significant beneficial effect was seen in trials that disproportionally enrolled male patients. This is consistent with lower folate levels, higher homocysteine levels, and higher incidence of stroke in men compared to women.[18-20] Folate deficiency or impaired folate metabolism likely underlies cardiovascular dysfunction while homocysteine may be an innocent bystander with hyperhomocysteinemia simply serving as

a biomarker of folate deficiency.[21] However, further studies are needed to evaluate the potential benefits of folate supplementation in primary stroke prevention, especially when combined with B vitamins.

Synthetic folic acid is one of the most common supplements. Clinical pharmacokinetic and pharmacodynamic studies on synthetic folic acid supplementation revealed that approximately 86% of folic acid in the hepatic portal vein is unmetabolized.[12,22] Synthetic folic acid must be converted to 5-MTHF to be biologically active.[12] Folate metabolism is impaired in patients with mutations in the methylenetetrahydrofolate reductase (*MTHFR*) gene encoding MTHFR, which catalyzes conversion of 5,10-methylenetetrahydrofolate to biologically active folate, 5-MTHF.[9] The most common *MTHFR* gene mutation characterized by C-to-T substitution at bp677 leads to a 60% reduction in MTHFR enzyme activity and subsequent folate deficiency.[9] Impaired folic acid metabolism in individuals with the *MTHFR* TT gene polymorphism leads to folic acid accumulation and folate deficiency and attenuates folate-mediated physiologic functions. Approximately one fourth of the global population are carriers of *MTHFR* 677C>T gene mutation, which occurs in nearly half of Hispanics.[23,24] Unmetabolized folic acid can accumulate and compete with natural folate for the folate transporter and the folate receptor, thereby reducing generation of biologically active folate.[12]

To examine the possible relevance of folic acid for stroke prevention, researchers at the China Kadoorie Biobank Study Group[25,26] analyzed genetic data from 156,253 participants in their study population who had a genetic variant in *MTHFR* which was associated with higher homocysteine levels. Among the 156,253 participants studied, 12,240 developed a stroke over a 12 year period. Individuals with *MTHFR* variant had a 13% higher risk of total stroke (adjusted OR: 1.13, 95% CI: 1.09 – 1.17), suggesting a link between active folate levels and risk of stroke.

The efficacy of folic acid supplementation in improving cardiovascular outcomes in hypertensive patients is variable due to differential expression of the *MTHFR* gene. In the China Stroke Primary Prevention Trial,[27] a total of 20,702 Chinese hypertensive patients were randomized to daily treatment with a single-pill combination of enalapril and 800 μg folic acid versus enalapril alone. Folic acid supplementation led to significant risk reduction in the first stroke (2.7%), first ischemic stroke (2.2%), and composite cardiovascular events (3.1%) compared to enalapril alone (3.4%, 2.8%, and 3.9%, respectively). However, the efficacy of synthetic folic acid supplementation in reducing risk of first stroke was markedly diminished in patients carrying the CT and TT gene polymorphisms compared with those with CC genotype. The authors posit that individuals with the TT genotype may require a high dosage of folic acid supplementation to overcome biologically insufficient levels. However, low-dose folic acid supplementation (400 μg daily) improved biochemical and physiological indicators of vascular function in patients with coronary artery disease, and high-dose folic acid supplementation (5 mg daily) provides no additional benefit.[28,29] Therefore, we propose supplementation with 5-MTHF to bypass folate metabolism and overcome deficiency of active folate in hypertensive individuals carrying the TT genotype. This could reduce risk of first stroke while avoiding potential risks associated with synthetic folic acid accumulation outlined below. Supplementation with 5-MTHF is supported by is supported by data from a cross-sectional analysis of Chinese hypertensive participants in the Chinese Stroke Primary Prevention Trials that revealed inverse association between serum 5-MTHF and homocysteine when 5-MTHF was ≤10 ng/mL.[30] This underscores the need for higher serum 5-MTHF status in hypertensive patients regardless of *MTHFR* genotype. Clinical trials are urgently needed to evaluate the efficacy of 5-MTHF for primary stroke prevention while avoiding potential risks associated with synthetic folic acid. Emerging noninvasive measures of central nervous system perfusion including retinal vascular imaging will likely play an important role in monitoring the effectiveness of stroke prevention strategies such as fortification or supplementation with synthetic folic acid versus 5-MTHF.[31]

3. Potential risks of supplementation with synthetic folic acid

Supplementation with synthetic folic acid can lead to accumulation of unmetabolized free folic acid (UMFA), especially in patients with CT or TT genotype for *MTHFR*. UMFA accumulation has been implicated in the development of chronic disease including colorectal cancer. In the Aspirin/Folate Polyp Prevention Study using folic acid as a chemopreventive agent,[29,32] subjects supplemented with 1 mg daily folic acid had more advanced lesions and multiple adenomas at 5-year follow-up. The folic acid group also exhibited a higher rate of invasive prostate cancer. The effects of supranormal folic acid supplementation have been attributed to decreased markers of cell differentiation and increased cell turnover associated with high concentrations of folate in colon cancer cells.[29,33] Folic acid supplementation and impaired folic acid metabolism have also been associated with immunosuppression,[34] cancer,[35] and cognitive impairment or dementia.[36] Therefore, folic acid supplementation poses

greater risks in certain populations including those with prior adenomas and vitamin B_{12} deficiency.

4. Conclusion

CVD is a global health challenge, with hypertension as a key risk factor. Folic acid supplementation has been shown to reduce risk of stroke with an associated reduction in homocysteinemia in hypertensive patients. However, the cerebrovascular benefits of folic acid are negligible in patients expressing the *MTHFR* TT gene polymorphism due to reduced MTHFR enzyme activity, impaired folic acid metabolism, and persistent active folate deficiency. Furthermore, accumulation of unmetabolized folic acid raises the specter of folic acid toxicity including increased risk of cancer, immune suppression, and dementia. Direct provision of biologically active folate, 5-MTHF, may lower the risk of first stroke and cerebrovascular events independent of *MTHFR* gene polymorphisms. Further research is urgently needed to test the efficacy of 5-MTHF in reducing cerebrovascular risk in those carrying the *MTHFR* TT genotype. A more personalized approach comprising genetic testing for *MTHFR* gene polymorphisms to guide supplementation with folic acid versus 5-MTHF may enhance stroke prevention and help reduce the global impact of CVD. However, such an approach is expensive and not routinely available, especially in low-income countries. Hence, the alternative approach of supplementing all patients, irrespective of their *MTHFR* genotype, with active folate instead of synthetic folic acid merits examination. It remains to be examined whether supplementation with active folate such as tetrahydrofolate or 5-MTHF is associated with the increased risk of cancer, immunosuppression, and dementia that have been reported with synthetic folic acid supplementation. Considering the growing epidemic of CVD and stroke, it is imperative that large, well-designed clinical trials are conducted to examine the effects of synthetic folic acid versus active folic acid on CVD prevention and outcomes.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

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Ethics approval and consent to participate

Not applicable.

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ACCSCIENCE PUBLISHING

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BRIEF REPORT

Assessment of ocular neuropathic pain following vitreoretinal surgery using 23-gauge sclerotomy

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Abstract

Ocular neuropathic pain refers to persistent post-operative perception of ocular discomfort in the absence of painful stimuli. This study investigates persistent ocular pain following 23-gauge pars plana vitreoretinal surgery. In the present study, patients who underwent either 23-gauge vitrectomy or silicone oil removal, under local or general anesthesia, were included. The symptoms of ocular neuropathic pain were evaluated using the brief pain inventory questionnaire before and 2 months after surgery. In addition, the impact of reported ocular symptoms on quality of life was assessed. We also evaluated the correlation between ocular pain and factors such as patient demographics and underlying systemic conditions. This study includes 75 eyes of 75 patients with an average age of 58.93 ± 12.05 years. Of the included patients, 31 (41.3%) were female. Among the participants, 67 (89.3%) underwent pars plana vitrectomy, and 8 (10.7%) experienced silicone oil removal surgery. Analysis using paired *t*-test or Wilcoxon signed-rank test, based on data normality, indicated no significant change in eye pain scores 2 months after surgery. However, the percentage of patients using analgesics increased from 4% before surgery to 17.3% 2 months after surgery ($P = 0.021$). Furthermore, based on a linear regression model, patients who reported increased analgesic usage 2 months after surgery also scored worse on the quality-of-life questionnaire ($P < 0.05$). We also found that those who reported ocular pain, facial pain, and photophobia before surgery had a higher likelihood of using analgesics after surgery ($P = 0.03$, 0.003, and 0.001, respectively). In addition, regression analysis revealed that patients with migraine headaches and lower levels of education were more likely to develop eye symptoms postoperatively ($P = 0.017$ and 0.044, respectively). In conclusion, surgeries involving 23-gauge scleral incisions do not significantly induce ocular neuropathic pain within 2 months after surgery. However, there is an observed increase in the use of analgesics following surgery.

Keywords: Chronic pain; Post-operative pain; Eye pain; Quality of life; Surveys and questionnaires; Vitrectomy

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1. Introduction

According to the International Association for the Study of Pain, pain is defined as an unpleasant sensory and emotional experience associated with or bears resemblance to actual or potential tissue damage.[1] Nociceptors play the main role in neural encoding of noxious stimuli such as trauma, surgery, or inflammation which damage nerve terminals. Acute nociceptive pain is a subjective perception with a direct link to consciousness, arising from a complex process in the central nervous system. Under some pathologic circumstances, a pain that continues to be perceived beyond the healing process or even exacerbates after surgery/tissue injury is called neuropathic pain.[2]

Persistent post-operative perception of ocular discomfort in the absence of painful stimuli is called ocular neuropathic pain. In such situations, patients experience pain, foreign body sensation, tearing, or photophobia in response to benign stimuli such as blinking. This is called allodynia, which is one of the features of ocular neuropathic pain.[3] Patients' symptoms outweigh objective signs of ocular surface defects such as cornea staining and/or conjunctival injection.[4-7] Altered nerve ending signaling following collateral injury to the branches of the trigeminal nerve during surgery is postulated as the main predisposing factor. With surgical trauma, even the density of goblet cells in the conjunctiva may change.

Neuropathic ocular pain has overlapping symptoms with dry eye syndrome and has sensory, psychic, cognitive, and behavioral elements.[3] Patients with genetic predisposing factors as well as those with fibromyalgia or psychiatric diseases such as depression are more susceptible to developing persistent post-operative pain.[8,9] Similar to dry eye syndrome, it is more common in females and older populations.[5,10]

Well-known ocular surgeries associated with ocular neuropathic pain include laser refractive surgery and cataract surgery.[5] However, few studies evaluated retinal surgeries in this regard. In this study, we aimed to evaluate the frequency of symptoms of ocular neuropathic pain after vitreoretinal surgeries involving a 23-gauge sclerotomy. We have also investigated the effect of different variables such as demographic factors and underlying conditions on post-operative symptoms.

2. Materials and methods

This observational study was conducted on patients who underwent 23-gauge (23G) sclerotomy incisions including pars plana vitrectomy and silicone removal. The study was conducted in a tertiary educational hospital from June 2021 to January 2022. The study protocol and procedures adhered to the principles outlined in the Declaration of Helsinki and were approved by the Tehran University of Medical Science's Institutional Review Board (Ethic Code: IR.TUMS.IKHC.REC.1397.162). Informed consent was obtained from each participant before inclusion in the study.

Following thorough ophthalmic examination, including slit-lamp (Haag-Streit, Mason, OH, USA), dilated fundus examination, and intraocular pressure measurement using the Goldmann applanation tonometer (AT-900; Haag-Streit AG, Koniz, Switzerland) mounted on the slit-lamp microscope, patients were scheduled for the appropriate surgery. Patients with difficulty in communication, a history of psychiatric, cerebrovascular, or neurologic disease, alcohol consumption, or using illicit drugs, analgesics, or opioids within the past 48 h of the surgery were excluded from the study.

In the operating room, patients were prepared for the surgery with local or general anesthesia. For local anesthesia, a combination of 0.02 mg/kg midazolam and 1 mcg/kg fentanyl was administered for intravenous sedation; following blood pressure monitoring, peribulbar injection of lidocaine 2% and marcaine was performed. For general anesthesia, midazolam 0.02 mg/kg, fentanyl 2 mcg/kg, atracurium 0.5 mg/kg, and propofol 2 mg/kg were employed. Two to three scleral incisions were conducted 3 to 4 mm posterior to the limbus on inferotemporal, superonasal, and superotemporal sites. In occasional cases when a watertight seal of the sclerotomy site was not achieved at the conclusion of surgery, Vicryl 8-0 or 7-0 absorbable sutures were placed. All surgeries were performed by two experienced vitreoretinal surgeons (N.E. or M.I.). All patients also underwent slit-lamp examination and intraocular pressure measurement on day 1, week 1, month 1, and month 2 after the surgery.

We assessed persistent pain in patients using the Persian version of the standardized brief pain inventory (BPI) questionnaire,[11] immediately before surgery and 2 months after surgery. BPI is a multidimensional measurement tool, which has been validated in the Iranian population.[11] A body figure is presented to the patient, on which the site of pain can be marked. The questionnaire has an 11-point numeric scale; the "0" corresponds to no symptoms, and the "10" indicates the worst imaginable symptom. The following items were evaluated: Deep eye pain, irritation, photophobia, tearing, foreign body sensation, deep head and facial pain, and numbness. The average of all items, defined as the overall eye pain score, was calculated to evaluate the correlation of ocular manifestations with demographic characteristics and systemic conditions. BPI questionnaire also evaluates the interference of symptoms

with various domains of personal life such as general activity, mood, relationships, sleep, enjoyment of life, the ability to walk, and the ability to perform daily work both outside and inside the home. The patients rated them through an 11-point numeric scale ("0" corresponds to no interference, and "10" shows complete interference).

The effect of demographic characteristics (such as patient's age, gender, and level of education), as well as background systemic conditions such as diabetes mellitus, hypertension, chronic back pain, musculoskeletal pain, migraine headaches, depression, type of surgery, and method of anesthesia on reported ocular symptoms and quality of life were investigated. The level of education of participants was classified into four levels: illiterate, not completed high school, with a high school diploma, and with a college degree.

The collected data were analyzed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA). The normal distribution of quantitative variables was evaluated with the Smirnov–Kolmogorov test. Paired *t*-test or Wilcoxon test was used to compare the alteration of each score after surgery based on the normality of the variables. To determine the distribution of categorical factors, the Chi-square test was applied. The regression model was used to determine the association of various predicting factors with post-operative overall eye pain score. To evaluate the association of a covariate with a dichotomous dependent variable, logistic regression analysis was employed. The significance level of the test was set at 0.05.

3. Results

A total of 75 eyes of 75 patients, including 31 (41%) females, were enrolled in this study. The average age of participants was 58.93 ± 12.05 (range: 35 – 82) years. Sixty-seven (89.3%) patients underwent 23G vitrectomy, and 8 (10.7%) patients had 23G silicone oil removal surgery. Sixty (80%) patients (52 patients underwent vitrectomy and eight patients had silicone removal surgery) received regional anesthesia (subtenon or retrobulbar block), and 15 (20%) (underwent 23G vitrectomy) received general anesthesia.

The distribution of the level of education of participants was as follows: 8% were illiterate, 29.3% did not complete high school, 29.3% had a high school diploma, and 9.3% had a college degree. The underlying condition at baseline included 28 patients diagnosed with diabetes mellitus (37.3%), 30 patients with hypertension (40%), 29 patients with chronic back pain (38.7%), 29 patients with musculoskeletal pain (38.7%), 11 patients with migraine headache (14.7%), and three patients with depression (4%). There were patients with more than one underlying condition at baseline. No post-operative complications such as uveitis, corneal epithelial defect, punctate epithelial erosion, significant ocular surface inflammation, or conjunctival injection were observed during follow-up examinations.

The prevalence and intensity of pain-related symptoms in eyes, as well as headache and facial numbness, were compared before and after surgery. Figure 1 depicts the individual scatterplot of each score before and after surgery. Although the frequency of some symptoms such as deep eye pain, photophobia, and facial numbness increased after surgery, the difference between patient-reported pain scores and other symptoms' scores before and after surgery was not statistically significant (Table 1). Likewise, the quality-of-life scores at baseline and 2 months following surgery did not show a significant change (Table 2).

Overall, eye symptoms at 2 months after surgery were not correlated with age and sex, but significantly varied among different levels of education (illiterate: 0.92 ± 0.74; not completed high school: 1.40 ± 1.36; high school diploma: 1.63 ± 2.58; college degree: 0.17 ± 0.44; $P = 0.044$). The presence of systemic diseases such as diabetes (mean pain score: 1.52 ± 2.36), musculoskeletal pain (mean pain score: 1.06 ± 1.63) and depression (mean pain score: 1.39 ± 2.12) was not associated with the overall eye pain scores in the post-operative period. However, a history of migraine headaches could increase the overall post-operative eye pain score (B: 1.35; 95% CI: 0.25 – 2.45; $P = 0.017$). The method of anesthesia (local or general) and type of surgery (vitrectomy or silicone oil removal surgery) did not affect eye symptoms 2 months after surgery.

Using systemic analgesics like non-steroidal anti-inflammatory drugs (NSAIDs) for eye symptoms increased from 4% at baseline to 17.33%, 2 months after surgery ($P = 0.021$). We found a weak positive correlation between post-operative analgesic usage and pre-operative eye pain score, photophobia, and face pain, but such significance was not found with foreign body sensation and tearing (Table 3).

In a subgroup analysis, patients who had analgesic consumption 2 months after surgery scored worse in items of quality-of-life questionnaire. They reported a significantly higher rate of disturbance in general activity, mood, normal work, relationship with others, sleep, and life enjoyment compared to those who were not using analgesics postoperatively (Table 4).

4. Discussion

In the present study, we showed that 23-gauge sclerotomy did not alter ocular pain scores significantly. Among the

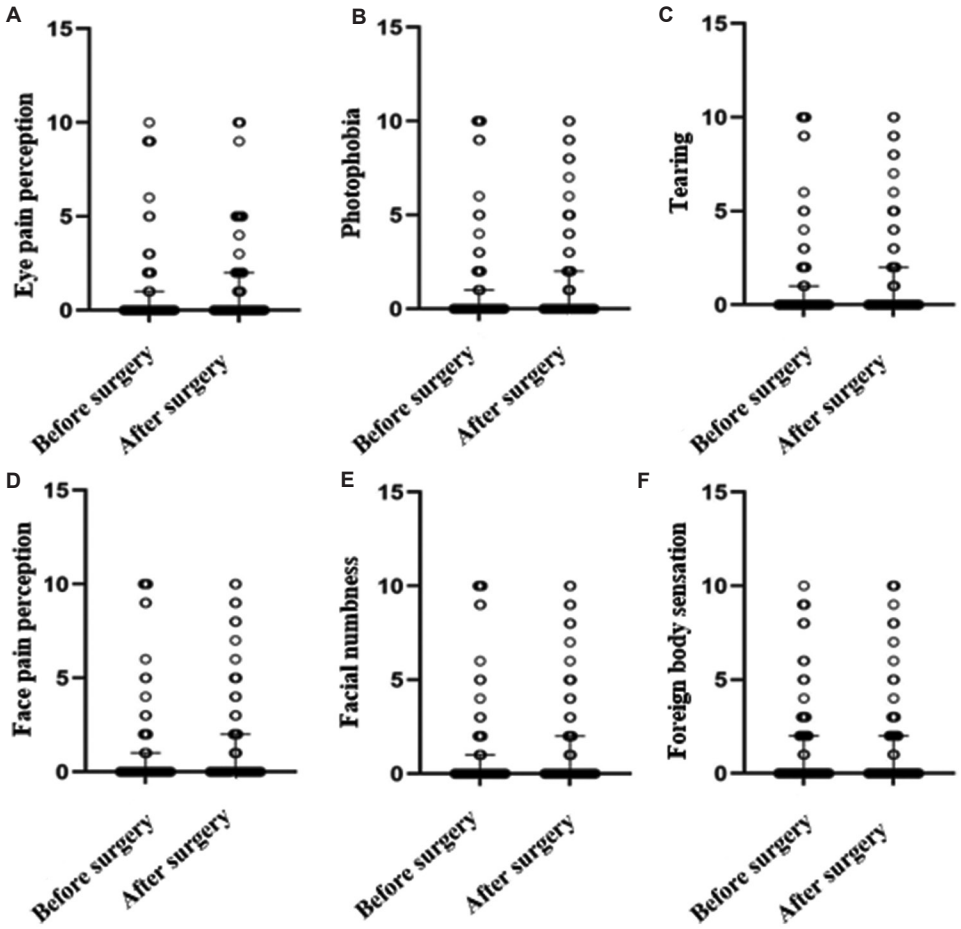

Figure 1. Individual scatterplots of symptoms of ocular neuropathic pain before and after surgery. No significant alternation was observed in eye pain (A), photophobia (B), tearing (C), facial pain (D), facial numbness (E), and foreign body sensation(F)

baseline characteristics factors, only the level of education and history of migraine could predict post-operative overall pain score. In addition, we observed a significant increase in analgesics consumption 2 months following surgery. Furthermore, patients using analgesics after surgery have reported worse scores on quality-of-life-related items. Patients who reported ocular and facial pain as well as photophobia before surgery were more likely to consume analgesics after surgery. Severing the enriched nerve plexus in surgeries involving corneal incisions has been shown to induce ocular neuropathic pain. However, few studies exist that investigate ocular pain following surgeries involving solely scleral incisions.[12,13]

Compared to the cornea which has the highest nerve density in the body, the sclera is less sensitive due to its lower nerve density and the less exposed nerves which are located far from the surface.[14] The anterior sclera is innervated by two long posterior ciliary nerves which are branches of the ophthalmic division of the trigeminal nerve.[15] These nerves pierce the sclera adjacent to the optic nerve and travel anteriorly to around the ciliary body. The small-gauge sclerotomies can injure these nerve endings. In addition, conjunctival manipulation, desiccation, exposure to microscope light, and toxicity of povidone-iodine or post-operative topical drugs can induce ocular surface abnormalities. A study conducted in 2013 investigated ocular pain after a conventional 20-gauge pars plana vitrectomy and reported that the frequency of ocular pain increased from 4.6% before surgery to 49.4% the day after surgery which then gradually decreased. In this study, 12.6% of patients reported persistent ocular pain at the final report which was done 2 months after surgery. Symptoms of foreign body sensation were found in 25% of patients.[12] The insignificant alteration of pain scores in our study may be related to the use of a smaller-gauge device to pierce the sclera with the induction of fewer nerve endings destruction. Interestingly, a retrospective study comparing 20-gauge versus 23-gauge vitrectomy showed that the 23-gauge vitrectomy group experienced less discomfort of the eye (0.96 weeks vs. 2.4 weeks), less pain and sleep disturbance, and consumed

Table 1. Prevalence and intensity of eye symptoms, patient-reported pain score, and other symptoms before and after surgery

Symptoms	No symptoms n (%)	Mild[c] n (%)	Moderate[d] n (%)	Severe[e] n (%)	Mean score (mean±SD)	*P*-value (pre and post)
Eye pain						
Pre-operative[a]	56 (74.6)	11 (14.7)	3 (4)	5 (6.7)	1.12±2.50	0.257
Post-operative[b]	50 (66.7)	13 (17.3)	8 (10.7)	4 (5.3)	1.34±2.55	
Photophobia						
Pre-operative	54 (72)	10 (13.3)	4 (5.3)	7 (9.4)	1.42±2.97	0.816
Post-operative	52 (69.3)	10 (13.3)	6 (8)	7 (9.4)	1.45±2.76	
Eye irritation and foreign body sensation						
Pre-operative	48 (64)	16 (21.3)	5 (6.7)	6 (8)	1.52±2.65	0.967
Post-operative	49 (65.3)	15 (20)	4 (5.3)	7 (9.4)	1.52±2.75	
Tearing						
Pre-operative	53 (70.7)	10 (13.3)	8 (10.7)	4 (5.3)	1.30±2.47	0.637
Post-operative	53 (70.7)	10 (13.3)	6 (8)	6 (8)	1.33±2.53	
Deep head and face pain						
Pre-operative	60 (80)	4 (5.3)	3 (4)	8 (10.7)	1.30±3.03	0.930
Post-operative	60 (80)	3 (4)	4 (5.3)	8 (10.7)	1.26±2.90	
Facial numbness						
Pre-operative	65 (86.7)	0	0	10 (13.3)	0.13±1.15	0.785
Post-operative	55 (73.3)	10 (13.3)	0	10 (13.3)	0.17±1.20	

Notes: [a]Pre-operative: Before the surgery; [b]Post-operative: Two months after the surgery. Eye symptoms: [c]Mild=1 – 3; [d]Moderate=4 – 6; [e]Severe=7 – 10. Data analyzed with paired *t*-test or Wilcoxon test based on data normality.

Table 2. Scores of quality of life at baseline and 2 months after surgery

Disturbance in items of quality of life	Pre-operative mean score (0 – 10) ± SD (range)	Post-operative mean score (0 – 10) ± SD	*P*-value
General activity	0.48±1.67 (0 – 10)	0.45±1.81 (0 – 10)	0.833
Mood	0.96±2.29 (0 – 10)	0.77±2.31 (0 – 10)	0.369
Walking ability	0.22±0.86 (0 – 6)	0.21±1.22 (0 – 10)	0.832
Normal daily work	0.49±1.64 (0 – 10)	0.46±1.79 (0 – 10)	0.789
Relationship	0.61±1.82 (0 – 10)	0.50±2.00 (0 – 10)	0.634
Sleep	0.60±1.77 (0 – 10)	0.54±1.98 (0 – 10)	0.654
Enjoyment of life	0.81±2.12 (0 – 10)	0.58±2.06 (0 – 10)	0.377

Note: Data analyzed with paired *t*-test or Wilcoxon test based on data normality.

Table 3. Association of pre-operative factors with post-operative analgesic use

Pre-operative variables	Post-operative analgesic use		Odds ratio (95% CI)	*P*-value
	No analgesic	Analgesic		
Eye pain	0.81±2.09	2.62±3.69	1.24 (1.02 – 1.51), B=0.219	0.030
Photophobia	0.82±2.00	4.31±4.80	1.35 (1.12 – 1.62), B=0.30	0.001
Foreign body sensation	1.29±2.49	2.62±3.18	1.17 (0.96 – 1.42)	0.112
Tearing	1.23±2.49	1.69±2.46	1.07 (0.85 – 1.33)	0.537
Face pain	0.77±2.32	3.85±4.56	1.28 (1.08 – 1.52), B=0.253	0.003

Note: Data analyzed with linear regression model.

Table 4. Association between post-operative disturbance of quality-of-life items and analgesic use

Post-operative disturbance in items of quality-of-life questionnaire	Analgesic use		*P*-value
	No analgesic	Analgesic	
General activity	0.15±0.75	1.92±0.84	0.021
Mood	0.16±0.77	3.69±4.35	<0.001
Normal work	0.10±0.47	2.23±3.83	0.002
Relationship with others	0.00±0.00	2.92±4.13	<0.001
Sleep	0.15±0.74	2.46±4.10	0.002
Life enjoyment	0.10±0.65	2.92±4.11	<0.001

lesser analgesics (19 patients compared to 64 patients in the 20-gauge group) 1 week after surgery.[13]

Although ocular pain scores did not alter significantly after surgery, a significant proportion of our patients used analgesics afterward. This subset of patients scored worse in the items of the quality-of-life questionnaire. In fact, analgesic consumption may have masked the ocular pain symptoms, in exchange for a decrease in quality of life. The previous studies have shown that the use of oral analgesics is an independent predictor of lower scores of quality of life.[16] Using NSAIDs is associated with fatigue, anxiety, and depression in patients with rheumatoid arthritis.[17] Based on our findings, higher scores of ocular and facial pain, as well as photophobia before surgery, herald higher odds of analgesic use after surgery. Detection of risk factors and appropriate management of post-operative ophthalmic pain may reduce the number of analgesic-dependent patients and may result in a more satisfactory outcome.

Although it is reported that the presence of underlying conditions such as diabetes mellitus, hypertension, musculoskeletal pain, and depression may affect the rate of reported pain symptoms,[8,9,18] we did not find any correlation between baseline and post-operative eye pain in this regard. The only underlying factors that could be considered predictive factors for post-operative eye pain were found to be migraine headaches and the level of education. It is known that in neuropathic pain syndromes, social, and psychiatric issues play a significant role in the perception of pain by the patients.[19]

The present study has several limitations. First, the sample size is relatively small. Second, we did not record the dose and frequency of analgesic used after surgery. Furthermore, we did not collect and analyze the data of intraoperative surgical steps such as endolaser photocoagulation or scleral indentation.

5. Conclusion

Most current studies have focused on the investigations of post-operative pain in different anterior segment surgeries, but reports surrounding pain after retinal surgery remain scarce. In this study, we evaluated ocular neuropathic pain in retina surgeries involving 23-gauge sclerotomies. Although the increasing trend of ocular pain at 2 months after scleral incisions was not significant, an increase in analgesic consumption after surgery, which affects the quality-of-life scores in this subset of patients, was observed. Considering the role of factors such as migraine headaches and the level of education in the prediction of post-operative pain can help physicians identify and inform patients susceptible to this condition. Future studies with larger samples and longer follow-ups are warranted.

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None.

Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The questionnaire and methodology for this study were approved by the Human Research Ethics Committee of Tehran University of Medical Sciences (Approval No: IR.TUMS.IKHC.REC.1397.162). Written informed consent was obtained from all participants included in this study.

Consent for publication

Written informed consent for publishing data was obtained from all participants.

Availability of data

Raw data of this study are available from the corresponding author on reasonable request.

Further disclosure

Part of this study's findings had been presented in the form of a poster at the CPS 42nd Annual Scientific Meeting, Montreal, Canada, on May 2022. A preprint version of this paper has been published (doi:10.21203/rs.3.rs-905460/v1), but the current article has undergone extensive revisions and improvements.

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ACCSCIENCE PUBLISHING

Global Translational Medicine

BRIEF REPORT

Recombinant human platelet-derived growth factor-BB-soaked gelatin sponge reduces patient pain in palatal graft donor sites

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Abstract

The free gingival graft (FGG) remains a gold standard for treating mucogingival defects and increasing the width of keratinized tissue. However, patients receiving FGG surgery report substantial post-operative discomfort, bleeding, and swelling, more so than those receiving connective tissue grafts, for example, and some of these post-operative sequelae can be difficult to manage. Various techniques are utilized in achieving hemostasis, but no current techniques effectively reduce the pain and discomfort to the patient. Ten FGGs were harvested from the palates of 10 patients to treat mucogingival deficiencies elsewhere in each patient. Immediately (<1 min) after harvesting the FGG, a gelatin sponge soaked in recombinant human platelet-derived growth factor-BB (rhPDGF-BB) was placed into the wound site and sutured in place with absorbable sutures. Patients were followed up on days 3, 7, and 14 for assessing pain levels utilizing patient-reported outcome measures (PROMs), which incorporate the visual analog scale and the quantification of analgesics consumed. Clinically, the rhPDGF-BB/gelatin sponge-treated FGG palatal donor sites began to heal by day 3 of follow-up, and the healing was completed during the visit on day 14. No adverse effects, including swelling or bleeding, were observed at any of the post-surgical time points. According to the visual pain score, patients experienced minor discomfort, but no dissatisfaction was reported. Patients whose palatal FGG donor site was treated with a gelatin sponge soaked in rhPDGF-BB reported little or no post-surgery discomfort and low morbidity at the donor site within 2 weeks. These findings are in sharp contrast to the well-known substantial discomfort most patients experience following the harvesting of a palatal FGG. The data suggest that treating FGG palatal donor sites with rhPDGF-BB-soaked gelatin sponge can improve patient experience.

Keywords: Pain; rhPDGF; Tissue adhesives; Wound healing; Palate

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1. Introduction

Many approaches have been developed to increase the width of keratinized tissue (KT) and/or obtain root coverage. The free gingival graft (FGG) remains the gold

standard for increasing the width of KT.[1] However, one of the biggest challenges in post-surgical management is the typical donor site pain and morbidity following this kind of procedure. FGG procedures are associated with significantly more post-operative discomfort, bleeding, and swelling compared to connective tissue graft (CTG) procedures.[2] Several methods, such as gelatin scaffolds, gelatin sponges, oxidized cellulose, and medicinal plant extract, all with or without the addition of cyanoacrylate,[3] have been utilized to control patients' post-operative morbidity. The use of cyanoacrylate does not affect the pain experienced by the patients. A palatal sent (protected vacuum form) is one of the most commonly used methods.

Palatal stents have been widely used following FGG procedures, but rarely alone. A gelatin sponge is often placed in the donor site following surgery to establish hemostasis. For that, clinicians need to ensure that this dressing is stable and efficient enough to act like a bandage, to control pain, bleeding, and swelling, and ideally accelerate the healing process.[4]

Cyanoacrylates are commonly employed as an alternative or as an adjunct to sutures. They have been reported to have bacteriostatic and hemostatic properties. Although, cyanoacrylates have proven beneficial to help with palatal wound healing, Stavropoulou *et al.* reported that no statistical difference in post-operative pain has been observed whether cyanoacrylate tissue adhesive or sutures were used for the donor site of the CTG procedure.[5] In this randomized clinical trial (RCT), the main benefit of cyanoacrylate was the time saved, which was around 5 min.[5]

The use of biologics in dental indications is gaining momentum and importance. Platelet-rich fibrin (PRF) is one such biologic to be considered. However, most available data from clinical trials have failed to provide sufficient evidence that PRF provides statistically and clinically significant benefits.[6,7] PRF may help lower patient morbidity from the palatal donor following the FGG harvest.[8] A recent RCT compared four common approaches, namely, gelatin sponges with sutures (control), cyanoacrylate, PRF with sutures, and palatal stents, to assess which best improved the patient-reported outcomes in pain, painkiller pill consumption, bleeding, swelling, and willingness to undergo a similar procedure in the future. The study findings suggested no statistically significant differences between treatment groups, and all interventions tended to decrease pain perception compared to the control. However, none of the used methods could completely resolve pain until the 11th day of the study.[9]

Given that PRF appears to have a tendency, albeit not significant, to positively influence healing of palatal donor sites in FGG procedures, we hypothesized that recombinant human platelet-derived growth factor (rhPDGF-BB), including the commercialized product GEM 21S® (Lynch Biologics), in which the growth factor is present in a much higher concentration, may have a greater patient benefit. Growth factors such as rhPDGF-BB play a key role in tissue healing and regeneration. rhPDGF-BB regulates central events involved in wound repair, such as cell proliferation, cell homing and differentiation, and tissue revascularization.[10] rhPDGF-BB is the best documented and only growth factor that has been shown in clinical trials to have significant positive benefits on both bone regeneration (e.g., guided bone regeneration, alveolar ridge preservation, treatment of periodontal defects) and soft-tissue wound healing (e.g., gingival recession and skin wounds) with no significant adverse effects.[11] In addition, the clinical trials showed that the rhPDGF-BB contains specific products that are not associated with any adverse effects, providing some evidence that rhPDGF-BB is a safe material for surgical treatments.[12] It was also shown in a prospective, randomized controlled clinical trial that treatment with rhPDGF-BB reduced the pain and lessened the side effects compared with autogenous grafts.[13]

Thus, since the patient's pain perception should be considered a key determinant for treatment planning and patient acceptance of the treatment plan, this report aimed to examine the clinical effect of rhPDGF-BB on palatal wound healing, as assessed by the patient using a patient-reported outcome scores (PROMs) questionnaire incorporating the visual analog scale (VAS). We hypothesized that using rhPDGF-BB can reduce the discomfort and lessen the pain for the patients compared with using PRF and gelatin sponges without rhPDGF-BB.

2. Materials and methods

2.1. Study design and setting

This study included ten healthy, non-smoking patients, including five males and five non-pregnant females within the age range of 35 – 54 years, who had undergone surgical treatment using a rhPDGF-BB-soaked gelatin sponge at a private practice after obtaining their consent. In all cases, initial prophylaxis using ultrasonic instruments and serial polishing cups was performed before the procedure. All procedures were performed by the same operator (D.M.). Local anesthesia (2% lidocaine with 1:100,000 epinephrine) was administered before FGG harvesting. Before harvesting, an absorbable porcine gelatin sponge (SURGIFOAM, Ethicon, USA) was cut to match the dimension of the graft to be harvested. The sponge was then soaked in rhPDGF-BB solution (GEM21S, Lynch Biologics, Franklin, TN, USA) for ≥ 10 min (Figure 1).

All FGGs were performed in accordance with Sullivan and Atkins' description,[9] with all grafts being placed on a prepared periosteal bed. Graft thickness was standardized to approximately 1 – 1.5 mm, depending on the palatal tissue thickness, as identified through bone sounding before surgery. After harvesting the FGG, the absorbable porcine gelatin sponge soaked in rhPDGF-BB was placed to cover the denuded palatal wound and on the recipient site. Mild manual compression of the wound area was conducted, with caution exercised not to wring out all the rhPDGF-BB solution from the sponge. Afterward, the rhPDGF-BB-soaked sponge was sutured in place with compressive palatal polyglycolic acid (PGA) 4/0 absorbable sutures (Figures 2 and 3). All FGGs were then stabilized with suspensory periosteal sling PGA 4/0 absorbable sutures (Figures 2 and 3). The transplanted grafts covered the recessions successfully, and the harvested sites were healed nicely without complications.

The post-operative instructions given to patients included discontinuing tooth brushing and flossing around the surgical sites for the 2 weeks following surgery, avoiding the area with the tongue to minimize any movement of the sponge, and using mouthwash as an alternative for maintaining oral hygiene. A soft food diet was prescribed during the 1st week to avoid any mechanical trauma to the sites. A rescue medication (600 mg of ibuprofen) was prescribed. Patients were instructed to take the drug only when necessary. No packs or dressing materials were applied to the donor sites, and no palatal stents were provided either.

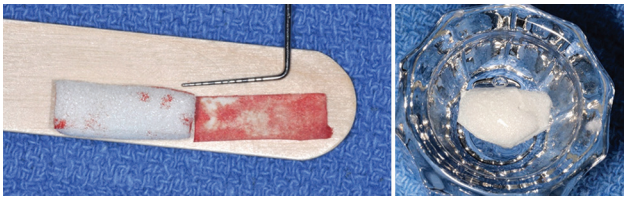

Figure 1. Photos showing the gelatin sponge cut to match the dimension of the graft to be harvested. The sponge was then soaked in rhPDGF solution for ≥10 min before being applied to the donor site.

2.2. Consecutive case series

Ten patients in need of an FGG were consecutively treated with the same technique as described below. All patients were requested to complete the PROMs questionnaire on days 3, 7, and 14 during the post-surgical follow-up. According to their survey responses, the patients reported continuous alleviation of discomfort, and they did not experience any pain in the 2nd week.

2.3. PROMs and clinical observation methods

Patients were followed up on days 3, 7 (±3), and 14 (±3) post-surgically to assess healing and record their pain scores. Patients were requested to make a mark on the VAS to denote the level of perceived pain. During follow-ups, all records were taken by the same clinician (D.M.).

3. Results

The recorded scores are presented in Table 1. Upon day 3, all patients reported mild (1 – 3) or no (0) pain. These patients had taken ibuprofen, which has been confirmed in a meta-analysis to be superior to acetaminophen in reducing pain.[14] No patients reported moderate or severe pain. During the follow-up on day 7, six patients reported no pain whatsoever, three reported only mild pain, one reported moderate pain, and no patients reported severe

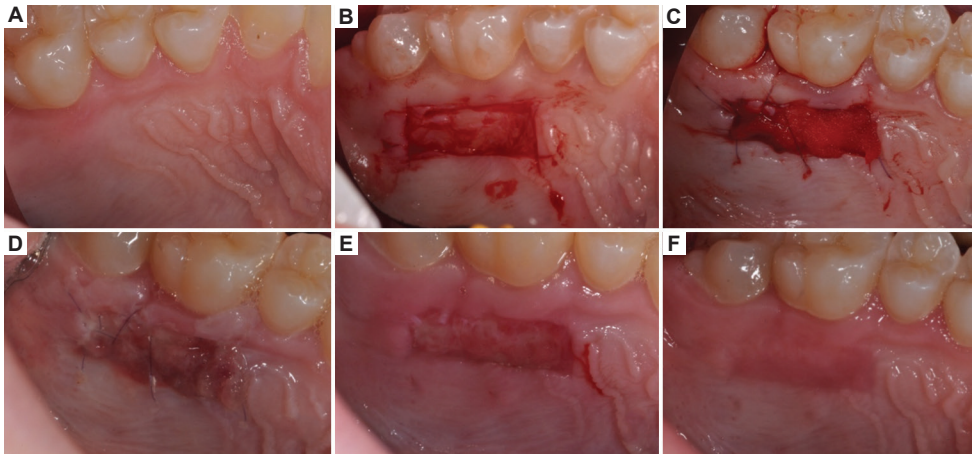

Figure 2. Photos showing the FGG harvesting, application of the rhPDGF-BB-soaked gelatin sponge to the donor site, and post-operative healing in patient 3. (A) Pre-operative situation; (B) FGG harvesting; (C) A pre-cut rhPDGF-BB-soaked gelatin sponge placed in the wound site and sutured with PGA sutures; (D) Donor site healing on day 3; (E) Donor site healing on day 8; and (F) Donor site healing on day 15.
Abbreviations: FGG: Free gingival graft; rhPDGF-BB: Recombinant human platelet-derived growth factor-BB; PGA: Polyglycolic acid.

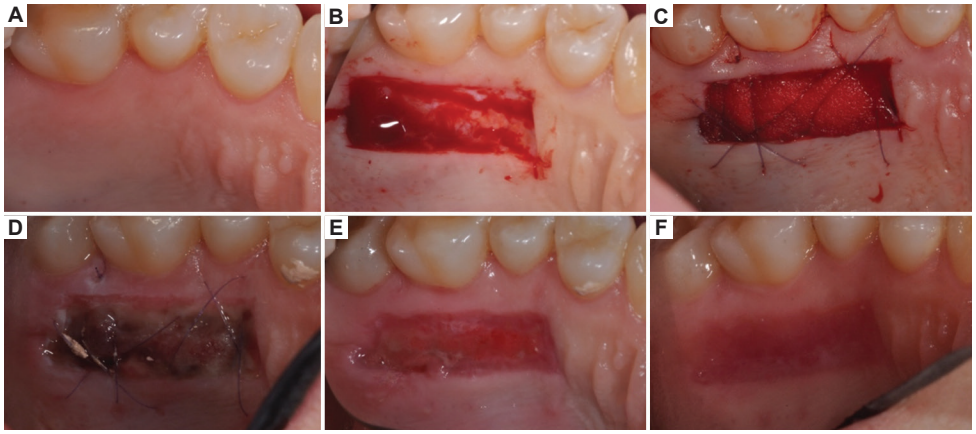

Figure 3. Photos showing the FGG harvesting, application of the rhPDGF-BB-soaked gelatin sponge to the donor site, and post-operative healing in patient 5. (A) Pre-operative situation; (B) FGG harvesting; (C) A pre-cut rhPDGF-BB-soaked gelatin sponge placed in the wound site and sutured with PGA sutures; (D) Donor site healing on day 3; (E) Donor site healing on day 10; (F) Donor site healing on day 13.
Abbreviations: FGG: Free gingival graft; rhPDGF-BB: Recombinant human platelet-derived growth factor-BB.

pain. During the visit on day 14, no patients reported any pain. Patients had an average VAS score of 2 at the day 3 follow-up, 1 at the day 7 follow-up, and 0 (i.e., no discomfort) at the day 14 follow-up (Figure 4). Clinically, the FGG palatal donor sites treated with rhPDGF-BB-soaked gelatin sponge had already started to heal on day 3. After 7 days, healing appeared well underway in the donor sites, which were completely healed on day 14 (Figures 2 and 3). No swelling or bleeding was observed at any of the post-surgical time points. Every patient was given a VAS, encompassing a range of perceived pain levels from "no pain" to "worst pain ever," during days 3, 7, and 14 visits for pain intensity assessment (Figure 4).

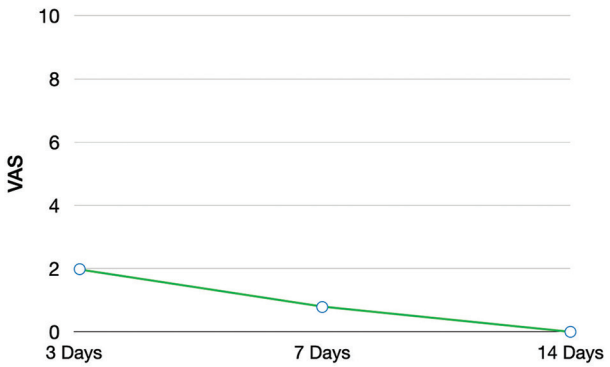

Figure 4. Alteration of patients' self-reported perceived pain level determined with visual analog scale

Table 1. The PROMs as indicated by VAS scores

Patient number	First visit (day 3)	Second visit (day 7)	Third visit (day 14 ± 3)
1	3	1	0
2	2	4	0
3	1	0	0
4	2	0	0
5	3	2	0
6	0	0	0
7	2	0	0
8	2	1	0
9	3	0	0
10	2	0	0

Abbreviations: PROMs: Patient-reported outcome measures; VAS: Visual analog scale.

4. Discussion

Post-operative pain is one of the most reported side effects following the FGG harvesting procedure.[2] In this survey, it is also the most common patient complaint following the procedure investigated in this study. Thus, reducing palatal donor site pain can significantly improve patient outcomes. This study reported that all 10 patients included experienced only mild pain three days after surgery and no pain during the day 7 follow-up, indicating the superiority of rhPDGF-BB-soaked gelatin sponge in improving patients' clinical outcomes as compared to other historical control methods such as stents, PRF, or gelatin sponge without rhPDGF-BB.

The key functions of placing a dressing in the donor site are attenuating pain and bleeding, making the healing process more comfortable, improving the patient experience, and supporting wound healing. Growth factors like rhPDGF-BB provide a positive stimulus on the mitogenesis (proliferation) and chemotaxis (homing) of the healing cells, expanding the number of healing cells in the wound. Likewise, rhPDGF-BB also increases

the deposition of collagen and glycosaminoglycans and promotes angiogenesis, which allows faster revascularization of the wound.

Compared to previous studies that prioritized the separate use of individual materials, this study innovated an alleviate means, combining rhPDGF-BB and gelatin sponge, to mitigate patient pain in the palatal graft donor sites. A clear mechanism of action of rhPDGF-BB promoting more closed wound healing[10] has been established in the literature, thus lending tremendous corroboration to our clinical observations.

The results of the present study are consistent with the original hypothesis that we set out to examine that is the use of a more concentrated rhPDGF-BB solution in a gelatin sponge would improve patient comfort compared to PRF or gelatin sponges without rhPDGF-BB, which had never been shown to mitigate pain intensity[11] as much as the method demonstrated in the present report. Randomized controlled trials comparing PROMs for patients treated with rhPDGF-BB-soaked gelatin sponges compared to gelatin sponges without rhPGF-BB are now warranted. Considering the limited sample size, lack of a control group, and treated sites, an additional number of subjects in a randomized controlled trial for investigating further intervention is recommended for further studies.

5. Conclusion

In summation, a swift placement of rhPDGF-BB-soaked gelatin sponges into palatal donor sites following FGG harvesting can result in improved patient-reported outcomes, less patient discomfort, and an overall patient post-operative experience.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

A signed written consent was obtained from each patient before the procedure.

Consent for publication

No patient identifiers were used in this report.

Availability of data

Data are available from the corresponding author on reasonable request.

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CASE REPORT

Targeted detection of Barrett's neoplasia: A case report

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Abstract

Barrett's esophagus (BE) is a precursor condition for esophageal adenocarcinoma (EAC). This case report describes the *in vivo* use of a fluorescently-labeled peptide heterodimer specific for epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER2) to identify residual neoplasia in a 52-year-old female patient with BE. This targeted contrast agent was topically administered after an incomplete endoscopic mucosal resection of high-grade dysplasia with carcinoma *in situ*. A flexible fiber-coupled multi-modal scanning fiber endoscope was used to capture near-infrared fluorescence images. This instrument was passed through the working channel of a standard upper endoscope for use as an accessory. Increased fluorescence intensity was observed from nodular mucosa as a real-time "red flag" to identify the presence of neoplasia. Pathologic tests were conducted on the resected tissues, confirming the presence of stage T1a EAC. The expression EGFR and HER2 was confirmed by immunohistochemistry *ex vivo*. These findings support integrated imaging as a potential strategy to detect Barrett's neoplasia.

Keywords: Barrett's esophagus; High-grade dysplasia; Esophageal adenocarcinoma; Peptide; Heterodimer; Epidermal growth factor receptor; Human epidermal growth factor receptor; Imaging

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1. Background

Barrett's esophagus (BE), which is a precursor condition for esophageal adenocarcinoma (EAC), is commonly found in patients suffering from chronic acid reflux. This metaplastic mucosa presents an increased risk of transforming into low-grade dysplasia (LGD), high-grade dysplasia (HGD), and EAC.[1] In the Western countries, EAC has been increasing rapidly in incidence[2] and is associated with a poor 5-year survival rate of approximately 20%.[3,4]

White light endoscopy (WLE) with random biopsies has been recommended by major medical societies for cancer surveillance in patients with BE.[5] However, this procedure

is time-consuming and limited by sampling error and is therefore not widely practiced by community physicians.[6] Pre-malignant lesions (LGD and HGD) are often flat in appearance and patchy in distribution.[7] Other methods, such as narrow-band imaging (NBI), offer enhanced sensitivity but less specificity, and present interpretation challenges, especially for patients with cancer, inflammation,[8] and excessive mucus or saliva. Despite several proposed classification systems, accurate characterization using NBI remains suboptimal, complicating its adoption in clinical practice.[9] Furthermore, confocal laser endomicroscopy provides real-time histologic imaging but its usage is constrained by high maintenance costs, a limited field-of-view,[8] and rigorous training requirements.[7,10]

A shift in molecular expression occurs in BE mucosa well in advance of gross morphological changes. Specifically, two cell-surface targets, namely epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER2), have been found to be highly overexpressed in esophageal neoplasia.[11] Detection of these targets using a wide-field imaging accessory may be a promising strategy for early cancer detection.

2. Case presentation

A 52-year-old female patient with BE presented for follow-up after an incomplete endoscopic mucosal resection (EMR) of HGD with carcinoma *in situ*. The patient had a history of sleeve gastrectomy. Routine upper endoscopy was performed, revealing mucosal changes in the distal esophagus, which were characteristically consistent with long-segment BE (Figure 1A). A maximum length of 4 cm was identified. A medium post-mucosectomy scar and surrounding residual nodular mucosa was found at the gastroesophageal junction consistent with a prior unsuccessful EMR. This finding motivated the application of a novel imaging strategy to identify any residual or missed lesions.

2.1. Clinical strategy

The targeted imaging strategy was performed to identify any missed lesions not seen with conventional WLE. A near-infrared (NIR)-labeled peptide heterodimer specific for EGFR and HER2 was topically administered to the Barrett's segment. Wide-field NIR fluorescence images were captured using the multi-modal scanning fiber endoscope (mmSFE) as an imaging accessory.[12,13] Fluorescence images of the nodular mucosa showed increased NIR fluorescence intensity (Figure 1B). A co-registered reflectance image was collected to identify mucosal anatomy (Figure 1C). The fluorescence and reflectance images were merged to produce a guiding sign for identifying early Barrett's neoplasia (Figure 1D). A specimen where the guiding sign was presented was excised from the mucosal region imaged. The specimen was evaluated and confirmed as EAC (stage T1a) by an expert gastrointestinal pathologist. Immunohistochemical test was also performed, validating the expression of EGFR and HER2 in the excised specimen.

2.2. Clinical findings

A single-channel therapeutic gastroscope (Olympus GIF-1TH190) was intubated and advanced into the distal esophagus of the patient. The lyophilized peptide heterodimer was reconstituted in 5 mL of normal saline and was then administered topically onto the BE mucosa using a standard spray catheter. After 5 min for incubation, the unbound contrast agent was washed away using an endoscopic irrigator. The mmSFE was inserted through the instrument channel to capture NIR fluorescence images. White light (WL) illumination was used concurrently to visualize the mucosal anatomy. EMR of the nodular mucosa and biopsies of the resection margin were performed. The tissues were submitted for routine pathological tests. Human use of the peptide heterodimer was regulated under IND #139,834 (sponsor DKT). The current study was approved by the Michigan Medicine IRB (HUM00158121) and was registered online at ClinicalTrials.gov (NCT03852576).

2.3. Diagnostic assessments

2.3.1. Peptide heterodimer

Specific peptide monomers for EGFR and HER2 were arranged in a heterodimer configuration and labeled with IRDye800, an NIR fluorophore[14,15] (Figure 2A). An E3

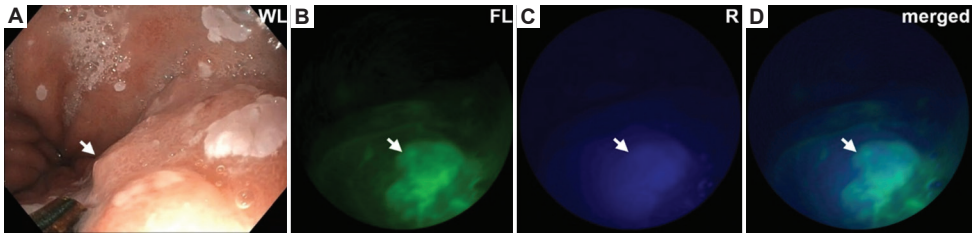

Figure 1. Targeted imaging. (A) High-definition white light image shows a nodular region of Barrett's esophagus (arrow). (B) Fluorescence image of the nodular region shows high intensity with a T/B ratio of 1.93 (arrow). (C) Co-registered reflectance image (R) provides surrounding mucosal anatomy. (D) The merged image provides a guide for therapeutic intervention with either endoscopic mucosal resection or biopsy.

linker was used to optimize the separation between the individual peptides to match the mean distance between these targets expressed on the mucosal surface. The fluorophore was chosen to have minimal spectral overlap with the WL illumination from a conventional endoscope (Figure 2B). Specific binding by the peptide heterodimer to EGFR and HER2 in Barrett's neoplasia was validated with tissue staining of human specimens *ex vivo*.[14] The NIR-labeled peptide heterodimer was synthesized as per the current good manufacturing practices and was lyophilized and aliquoted (1.8 mg) in 10 mL amber vials to protect them from light. The purity of the peptide heterodimer was determined as ≥95.0% by high-performance liquid chromatography, and its stability was assessed based on visual appearance, purity, and molecular weight.

2.3.2. Wide-field imaging accessory

An mmSFE was designed for clinical use as an imaging accessory. This flexible fiber-coupled instrument was passed forward through the working channel of a therapeutic gastroscope (Olympus GIF-1TH190). Excitation at λ_{ex} = 779 nm was delivered by a centrally located scanning fiber (Figure 3A). A ring of six collection fibers collected and delivered fluorescence and reflectance signals to detectors of a portable cart that is transported into the procedure room[12,13] (Figure 3B and C).

2.3.3. Image analysis

Reflectance and fluorescence signals were mapped onto the blue and green channels. A custom Chan–Vese algorithm[16] was used to segment the target from the region of interest (ROI) within video frames. The region around the target (30-pixel-wide) was considered the background region. The target-to-background (T/B) ratio calculation was performed by comparing the mean intensity of the target (T) and background (B) regions. The T/B ratios were used to classify the ROI as either positive (HGD/EAC) or negative (LGD/BE/squamous epithelium).

3. Discussion

A clinical imaging study was performed to evaluate the feasibility of utilizing a peptide heterodimer to detect early Barrett's neoplasia *in vivo*. A peptide heterodimer was labeled with IRDye800 to provide high image contrast from foci of disease. This fluorophore has spectral properties that avoid tissue autofluorescence and has

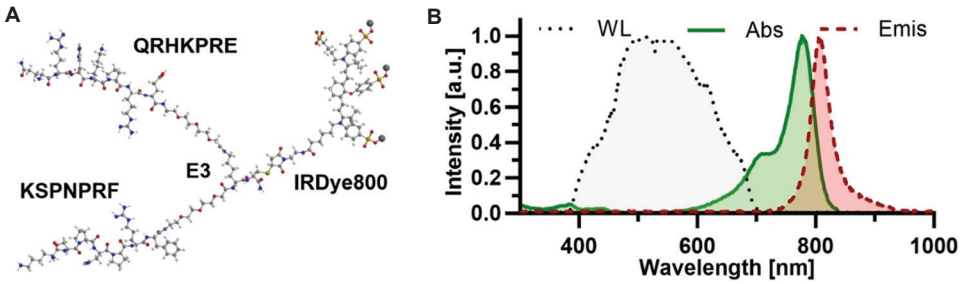

Figure 2. Near-infrared-labeled peptide heterodimer. (A) Biochemical structure is shown for the IRDye800-labeled peptide heterodimer. This targeted contrast agent consists of distinct peptide monomers QRHKPRE and KSPNPRF specific for EGFR and HER2, respectively. A E3 triethylene glycol linker was used to optimize the separation between the individual peptides. (B) The absorbance (Abs) and fluorescence emission (Emis) spectra for IRDye800 are shown. This fluorophore was chosen to minimize spectral overlap with the white light illumination from a conventional endoscope.

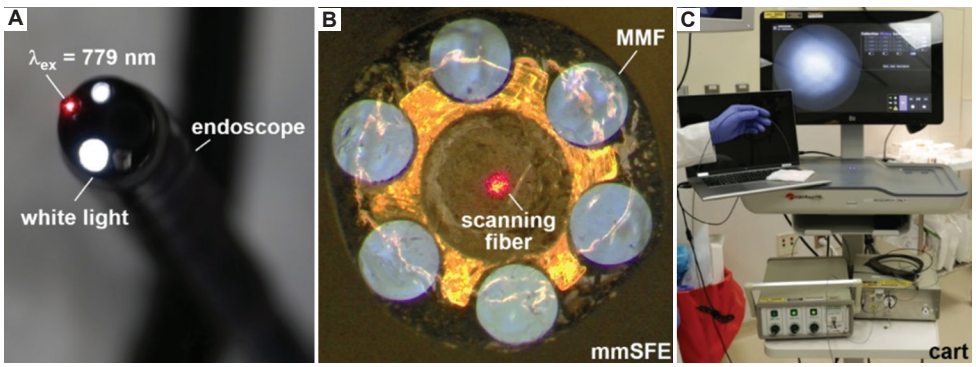

Figure 3. Wide-field imaging accessory. (A) The mmSFE has a rigid tip with dimensions of 9 mm in length and 2.4 mm in diameter and is shown passed forward through the instrument channel of a standard medical endoscope (Olympus #GIF-HQ190). (B) Excitation at λ_{ex} = 779 nm was delivered through a centrally located scanning fiber. Near-infrared fluorescence was captured by a ring of six multi-mode fibers. (C) The light source, detectors, and computer were contained on a portable cart that was transported into the procedure room.

minimal overlap with WL illumination. By comparison with alternate detection methods, our integrated imaging methodology allows for the simultaneous use of both WL and fluorescence imaging. This innovative approach enabled the identification of residual neoplasia in a BE patient who had undergone an incomplete EMR. This technology can also be used to improve visualization of flat and subtle pre-malignant lesions in BE patients who are at an increased risk of developing EAC. A larger clinical study of 31 human subjects showed that the mean T/B ratio for Barrett's neoplasia (HGD and EAC) was significantly greater than that for non-dysplastic BE, LGD, and squamous epithelium.[13] The T/B ratio was determined using a deep learning algorithm.[17] This methodological advantage provided high levels of sensitivity and specificity for the detection of Barrett's neoplasia, thus overcoming many shortcomings of conventional detection approaches.

Our findings contribute not only to the expanding endoscopic arsenal for the detection of Barrett's neoplasia but also advance targeted detection methods for other imaging methodologies. Peptide heterodimers can also be radiolabeled to stage metastatic Barrett's neoplasia using positron emission tomography-computed tomography to aid in identifying optimal treatment strategies for these patients. A future clinical study with a larger number of subjects studied at multiple clinical sites can be performed to further validate the initial findings and explore the full potential of this targeted approach for early detection of Barrett's neoplasia.

4. Conclusion

A peptide heterodimer was used to visualize the expression of EGFR and HER2 concurrently to identify early EAC (stage T1a) in a region of nodular Barrett's mucosa following a prior incomplete EMR. A flexible fiber-coupled mmSFE was used to capture *in vivo* images. This integrated imaging strategy demonstrates a promising approach for early detection of Barrett's neoplasia. Patient outcomes may be improved by earlier and more accurate identification of pre-malignant lesions.

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Conflict of interest

Jing Chen and Thomas D. Wang are inventors on patents filed by the University of Michigan on the peptide heterodimer used in the study. Eric J. Seibel is an inventor on patents filed by the University of Washington on the mmSFE. The remaining authors declare that they have no conflicts of interest.

Author contributions

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Data analysis: Tse-Shao Chang, Yang Jiang, Eric J. Seibel
Writing – original draft: Tse-Shao Chang, Thomas D. Wang
Writing – review & editing: Tse-Shao Chang, Thomas D. Wang

Ethics approval and consent to participate

The research study was approved by the Michigan Medicine IRB (HUM00158121) and was registered online at ClinicalTrials.gov (NCT03852576). The enrolled subject provided written informed consent.

Consent for publication

Permission was obtained from the subject to publish results.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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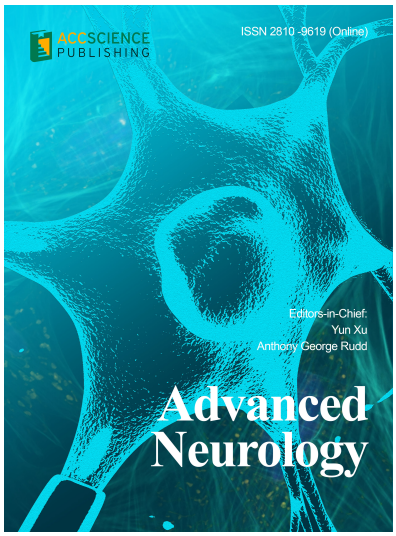

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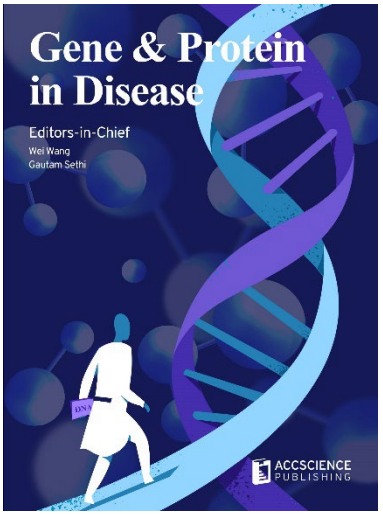

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