# Apoptoza u stijenci nativne vene u neuspjehu arteriovenskih fistula za hemodijalizu

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**UNIVERSITY OF ZAGREB**

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**To whom it may concern**

To my lovely family, Afrim, Lisa and Lend, and to my parents, who are the source of inspiration and encouragement in my life.

## LIST OF BOOKMARKS AND ABBREVIATIONS

| | |
|---|---|
| ACE- | angiotensin converting enzyme |
| AIDS | acquired immune deficiency syndrome |
| AIF | apoptosis inducing factor |
| AVF- | arterio-venous fistula |
| AVG- | arterio-venous graft |
| B/C- | brachial-cephalic |
| CVC- | central venous catheter |
| cfDNA- | cell free DNA |
| DAB- | 3,3'-diaminobenzidine |
| DNA- | deoxyribonucleic acid |
| EC- | endothelial cell |
| ePTFE- | expanded polytetrafluoroethylene |
| ESRD- | End-Stage Renal Disease |
| GSV- | great saphenous vein |
| HBsAg- | hepatitis B virus antigen |
| HCV- | hepatitis C virus |
| HD- | Hemodialysis |
| HE- | hematoxylin eosin staining |
| HIV- | human immunodeficiency virus |
| IL- | Interleukin |

| | |
|---|---|
| IHC- | Immunohistochemistry |
| IQR- | interquartile range |
| NIH- | neointimal hyperplasia |
| p- | the level of significance |
| R/C- | radial-cephalic |
| SMC- | smooth muscle cell |
| TUNEL- | terminal deoxynucleotidyl transferase dUTP nick end labeling |
| UCCK- | University Clinical Center of Kosovo |
| VSMC- | vascular smooth muscle cell |

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# 1. INTRODUCTION

## 1.1. Definition of apoptosis

The normal cell is confined to a fairly narrow range of function and structure by its genetic programs of metabolism, differentiation, and specialization; by constraints of neighboring cells; and by the availability of metabolic substrates. Generally, normal tissue homeostasis is characterized by a balance between proliferation and apoptosis. More severe physiologic stresses and some pathologic stimuli may bring about a number of physiologic and morphologic cellular adaptations, during which new but altered steady states are achieved, preserving the viability of the cell and modulating its function as it responds to such stimuli. A selective increase of proliferation leads to hyperplasia, or an increase in the sizes of individual cells, called hypertrophy. Conversely, atrophy is an adaptive response in which there is a decrease in the size and function of cells (1-4).

Apoptosis is defined as an active physiological and genetically controlled process of cell suicide that carries an important function in the development and homeostasis of multicellular organisms. Since its discovery, apoptosis emerged as a molecular control point in the regulation of physiological processes, toxic insults and diseases by means of programmed cell death. Apoptosis occurs normally during development and aging and as a homeostatic mechanism to maintain cell populations in tissues. It also occurs as a defense mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (5-7). It is a highly regulated and energy requiring process, controlled genetically and often elicited by endocrine signals (4-6). Apoptosis is the essential physiological mechanism for selective elimination of cells. It plays an integral part in a variety of biological events, including morphogenesis, the process of cell turnover and removal of harmful cells (8,9).

The process was recognized in 1972 by Kerr et al., by the distinctive morphologic appearance of membrane-bound fragments derived from cells, and named after the Greek designation for "falling off" (1,8,10,11).

The historical development of the cell death concept is reviewed, with special attention to the origin of the terms necrosis, coagulation necrosis, autolysis, physiological cell death, programmed cell death, chromatolysis (the first name of apoptosis in 1914), karyorrhexis, karyolysis, and cell suicide, of which there are three forms: by lysosomes, by free radicals, and by a genetic mechanism (apoptosis) (1,11,12,13).

Direct environmental stress and injury produce cell death by both necrosis and apoptosis (14). Although both necrosis and apoptosis result in cell death, they differ in several morphological and cellular regulatory features. Whereas necrosis is always a pathologic process, apoptosis serves many normal functions and is not necessarily associated with cell injury. Necrosis is characterized by the rapid loss of cellular homeostasis, rapid swelling as a result of the accumulation of water and electrolytes, early plasma membrane rupture, and the disruption of cellular organelles (Figure 1) (15). It induces an inflammatory response (4-6). Apoptotic cells break up into fragments, called apoptotic bodies, which contain portions of the cytoplasm and nucleus (10).

Although we emphasize the distinctions between necrosis and apoptosis, there may be some overlaps and common mechanisms between these two pathways. In addition, at least some types of stimuli may induce either apoptosis or necrosis, depending on the intensity and duration of the stimulus, the rapidity of the death process, and the biochemical derangements induced in the injured cell (3,16). When damage to membranes is severe, or absent sources of cell energy, lysosomal enzymes enter the cytoplasm and digest the cell, and cellular contents leak out,

resulting in necrosis (3). Some noxious stimuli, especially those that damage deoxyribonucleic acid (DNA), induce another type of death, apoptosis, which is characterized by nuclear dissolution without complete loss of membrane integrity.

Sloviter suggested that perhaps the terms necrosis and apoptosis should be redefined as "passive cell death" and "active cell death" (5,7). The mechanism whereby apoptosis is induced has recently gained attention as a possible treatment for a variety of diseases including excessive cell proliferation (17). However, apoptosis is also involved in a wide range of pathologic conditions, including acute neurological injuries, neurodegenerative diseases, cardiovascular diseases, immunological diseases, acquired immune deficiency syndrome (AIDS), and cancer (18). The process of apoptosis is controlled by various signals originating extracellularly or intracellularly. In living cells, apoptotic mitochondrial changes are predominantly prevented by anti-apoptotic Bcl-2 members (9,19). The duration of apoptosis is estimated to be from 12 to 24 hours, but in cell culture visible morphologic changes are accomplished in less than two hours (13). The process is under genetic control and can be initiated by an internal clock, or by extracellular agents such as hormones, cytokines, killer cells, and a variety of chemical, physical, and viral agents (6,12,19).

### 1.1.1. Morphologic and biochemical changes in apoptosis

Apoptosis is an active, genetically controlled type of cell death with distinct morphologic and biochemical features (20,21). It involves cell suicide in response to intrinsic signals (mitochondrial pathway) or extrinsic stimuli (death receptor pathway) in order to maintain homeostasis of the organism. Because apoptotic and necrotic stimuli both lead to mitochondrial

damage, this organelle appears to be a point of convergence of the pathways that mediate these morphologically distinct forms of cell death (1,6,19,22).

The cell death process is executed in an organized fashion reflecting the presence of well-preserved molecular pathways (23). The process may be divided into an **initiation phase**, during which some caspases become catalytically active, and an **execution phase**, during which other caspases trigger the degradation of critical cellular components. The initiation phase occurs from two pathways: **intrinsic (mitochondrial)** and **extrinsic (death-receptor initiated)** pathway (1,6,24).

The basic morphological characteristics of apoptotic cells and apoptotic bodies have been described on the basis of classic light microscopy of histologic hematoxylin-eosin (HE) stained slides. The apoptotic cell appears as a round or oval mass with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments (1,13,25). Morphology is characterized by:

- **Cell shrinkage**: the cell is smaller in size; the cytoplasm is dense, dark red; and the organelles, though relatively normal, are more tightly packed.
- **Chromatin condensation**: the chromatin aggregates peripherally, under the nuclear membrane, into dense masses of various shapes and sizes. The biochemical hallmark of apoptosis is degradation of DNA into nuclear fragments (20). The nucleus itself may break up, producing two or more fragments (karyorrhexis) (1,23).
- **Formation of cytoplasmic blebs and apoptotic bodies**: the apoptotic cell first shows extensive surface blebbing, then undergoes fragmentation into membrane-bound apoptotic bodies composed of cytoplasm and tightly packed organelles, with or without nuclear fragments, surrounded by light, “hollow space” (halo) (25).

- **Phagocytosis of apoptotic cells or cell bodies, usually by macrophages**: the apoptotic bodies are rapidly ingested by phagocytes and degraded by the phagocyte's lysosomal enzymes (Figure 1) (1,10,15,16,23,24).

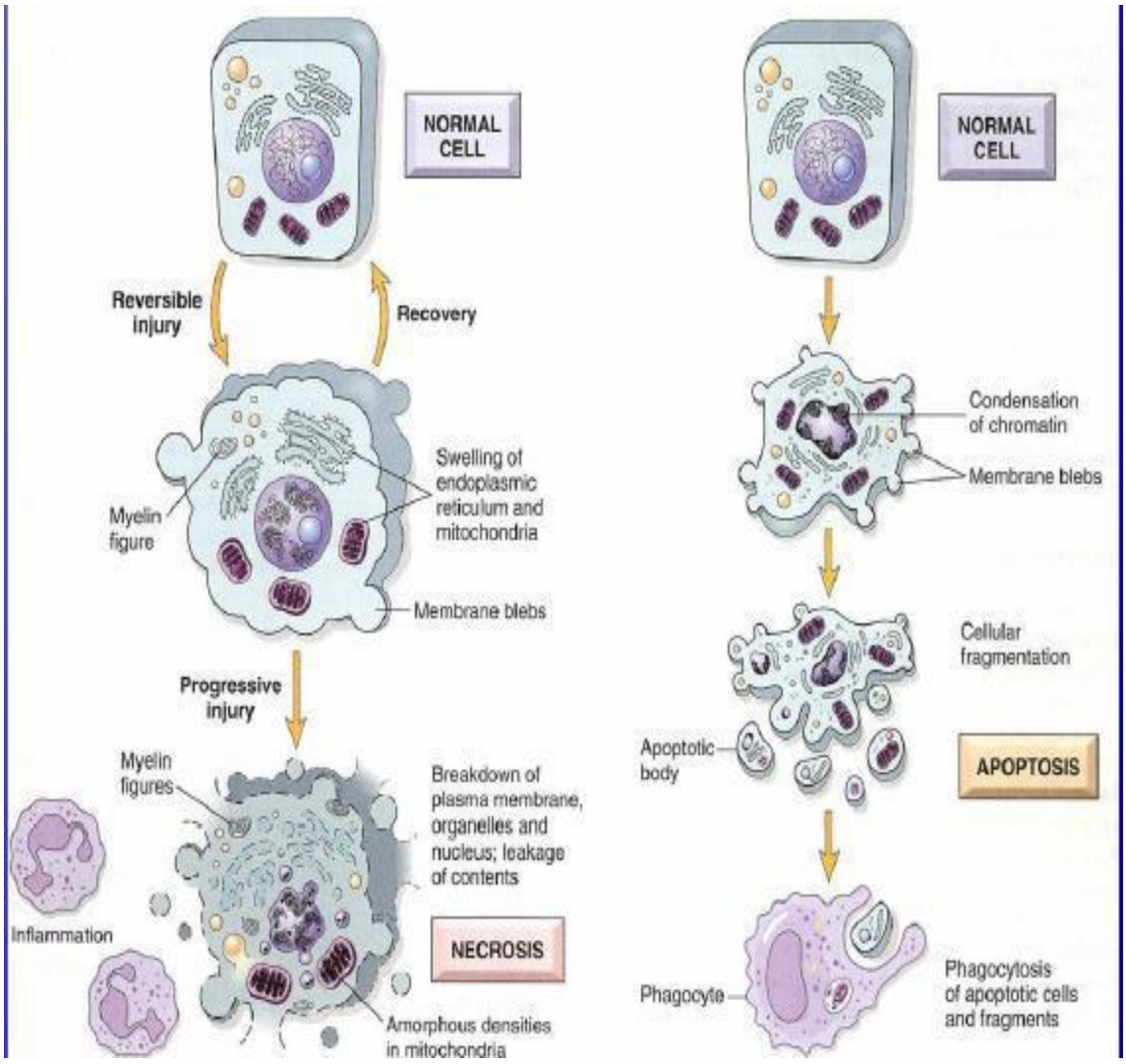



Figure 1. Schematic illustration of morphologic changes in cell injury culminating in necrosis or apoptosis adopted from Robins and Cotran (15)

Plasma membranes are thought to remain intact during apoptosis, until the last stages, when they become permeable to normally retained solutes (10,13,25).

Quantification of apoptosis should include enough microscopic fields and identification of the cell type undergoing apoptosis (13). Belicza showed that during more than 25 years of research into apoptosis, the Zagreb Group for the Study of Apoptosis (Apoptosis Section, Department of Basic Medical Sciences, Academy of Medical Sciences of Croatia) has found it possible to determine the number of apoptotic cells and apoptotic bodies (apoptotic index) in daily routine on classic HE stained histologic slides by counting in 10 large fields under light microscope, following the methodology of mitotic index determination (25).

### 1.1.2. Control and regulation of apoptosis

Apoptosis is now possible to prove in several ways: a) microscopic cell changes; b) on the basis of DNA fragmentation; c) proof of caspase; d) changes in the cell membrane; e) mitochondrial tests (5,7,12).

The apoptotic process is regulated by specific activation of a family of intracellular cysteine proteases known as caspases (cysteine aspartyl-specific proteases) inducible by various cellular and external stimuli. Two regulatory pathways are recognized. The intrinsic pathway (mitochondria pathway or type I) is regulated by apoptosis promoter proteins (such as Bax or Bcl2) and involves specific caspases (especially caspase 9) along with the participation of mitochondria. The extrinsic pathway (trans-membrane pathway or type II) is regulated by proteins such as Fas, the tumor-necrosis factor receptor (TNF-R) family proteins and involves specific caspases (especially caspase 8) connecting ligand binding at the cell surface to apoptosis induction (1,3,21,26).

**The Bcl-2** family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members. The Bcl-2 gene family

consists of >15 members (14,18). Krijnen et al. studied the expression of two such regulatory proteins, Bcl-2 and Bax, in the hearts of patients who died of acute myocardial infarction (3,5,18). No Bcl-2 was found in the infarcted area itself (5). The anti-apoptotic members of this family, such as Bcl-2 and Bcl-XL, prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm. After entering the cytoplasm, cytochrome c and AIF directly activate caspases that cleave a set of cellular proteins to cause apoptotic changes. In contrast, pro-apoptotic members of this family, such as Bax and Bak**,** trigger the release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm via acting on mitochondrial permeability transition pore, thereby leading to caspase activation (8,9,14).

Bcl-2 is a mitochondrial membrane protein which protects against a wide variety of stimuli which induce apoptosis. Bcl-2 family members can homodimerise or heterodimerise with other family members, such as pro-apoptotic proteins Bax and the balance of expression between family members can predispose or protect against apoptosis. Bcl-2 and Bax are homologous proteins that have opposing effects on cell life and death, with Bcl-2 serving to prolong cell survival and Bax acting as an accelerator of apoptosis. Bax is a member of Bcl-2 family and when overexpressed, accelerates apoptosis (5,9,27).

In recent years, it has been well established that Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death. A large number of Bcl-2-related proteins have been isolated and divided into three categories:

1. Anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1 (B£-1) and Boo, all of which exert anti-cell death activity and share sequence homology, particularly within four regions, Bcl-2 homology (BH) 1 through BH4, although some members lack an apparent BH4 domain.

2. Pro-apoptotic members such as Bax, Bak, Bad, Mtd (Bok), Diva, which share sequence homology in BH1, BH2 and BH3 but not in BH4, although significant homology at BH4 has been noticed in some members.

3. `BH3-only proteins', the pro-apoptotic proteins which include Bik, Bid, Bim, Hrk (DP5), Blk and Bnip3, Bnip3L, and share sequence homology only in BH3 (9).

Proteins of the Bcl-2 family together with mitochondria, cytochrome c, and caspases, have among others been identified as essential components of the intracellular apoptotic signaling pathways (14,18).

**p53** protein accumulates in cells when DNA is damaged, and it arrests the cell cycle to allow time for repair. However, if the damage is too great to be repaired successfully, p53 triggers apoptosis (24). p53 has been called a “cellular gatekeeper” by Levine in 1997, or “the guardian of the genome” by Lane in 1992, because of its central role in coordinating the cellular responses to a broad range of cellular stress factors (28). The p53 protein is expressed by all normal cells, but the half-life of the normal protein is so short (6 to 30 minutes) that it does not accumulate in levels high enough to be detected by standard immunohistochemical techniques (29-31). By contrast, mutant p53 protein has an extended half-life, accumulates, and is readily detectable in the cell nucleus. Alterations are extremely common in human cancer (32). Inactivation of the p53 tumor suppressor gene occurs in over half of all human tumors, implying that loss of this gene

represents a fundamentally important step in the pathogenesis of cancer (1,27). p53-related apoptosis is one of the regulatory mechanisms of vein wall homeostasis. During varicose vein development, its activation occurs in the early stages of the disease (30,33).

**Caspases** are cysteine proteases divided functionally into two groups: initiator (caspase 8, caspase 9) and executioner (caspase 3, caspase 6, caspase 7), depending on the order in which they are activated during apoptosis. In general, caspases form a key step in the process of apoptosis and serve as the central executioners of apoptosis (3,5,19,34). Although there are at least 14 caspases in humans, only a subset of these enzymes is detectably proteolytically activated by various distinct death stimuli in different cell types (35). Execution phase of apoptosis is mediated by a caspase 3 dependent pathway (10). Caspases are crucial mediators of programmed cell death (apoptosis) (6,35).

The significance of apoptosis has mostly been studied using the TUNEL assay (terminal deoxynucleotidyl transferase (TdT)-labeled dUTP nick labeling), a method described by Gavrieli et al. The TUNEL method detects DNA strand breaks in tissue sections and allows quantification and location of apoptotic cells by light microscopy, but is insufficient in distinguishing the different types of cell death (7,13,14,20).

### 1.2. Apoptosis in vessels

The formation of new blood vessels is an essential part of many physiological and pathological processes including embryogenesis, wound healing, formation of blood supply to tumors or ischemic tissue. Remodeling of a vessel describes an increase and decrease in its lumen size accompanied by changes in vessel wall area and components. One of the factors influencing

vascular wall development and remodeling is programmed cell death, which regulates tissue mass and its architecture. A number of studies have demonstrated programmed cell death in vessels that remodel postnatally, as a result of a balance between apoptosis and cell proliferation (11,30,36).

Vascular smooth muscle cells can perform both contractile and synthetic functions, which are associated with and characterized by changes in morphology, proliferation and migration rates, and the expression of different marker proteins. Because of the diversity among smooth muscle cells, blood vessels attain the flexibility that is necessary to perform efficiently under different physiological and pathological conditions (11,36,37). Apoptosis has been detected in atherosclerotic lesions of native coronary arteries, specimens from vein grafts, especially in restenotic lesions with high apoptotic index near 70%, varicose veins and other changes (2,14,38). Cell death occurs in both physiological and pathological contexts in the cardiovascular system. Physiological cell death is responsible for the sculpting and remodeling of the heart and blood vessels in response to the changing requirements of the tissues they supply (11,39).

### 1.2.1. Apoptosis in atherosclerotic disease

Atherosclerosis, the principle cause of heart attack, stroke and gangrene of the extremities, is responsible for more than a half of all mortality in Europe. The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall (40). Observations that cell death occurs in atherosclerosis have already been made by Virchow in 1858 (11).

The middle layer of the healthy vessel wall, the tunica media, contains an abundant population of vascular smooth muscle cells. During atherosclerosis, lipoproteins and cells accumulate in the

tunica intima, which is the innermost layer that separates the media from the lumen (41). Studies in human renal, coronary and carotid arteries have shown increased apoptosis and increased numbers of intrinsic pathway promoters including the apoptotic promoter Bax (26). Atherosclerotic lesions are characterized by regions displaying an excessive proliferation of the intimal tissue alterating with tissue death (4). As the main cellular components of the blood vessels, endothelial cells (EC) and smooth muscle cells (SMC) play central roles in vascular biology and pathology. Vascular endothelial cells are continuously exposed to a range of hemodynamic forces, which have a great impact on their cellular structure and function. The apoptotic death of smooth muscle cells could play a significant role in the aortic and plaque rupture. Massive induction of EC apoptosis in manipulated arteries can induce the formation of thrombus with appearances similar to eroded vessels, which suggests that EC apoptosis may trigger thrombosis (2,10,11,38,39).

It has been suggested that apoptosis contributes to the pathogenesis of atherosclerosis and to the instable plaque syndrome. Variations in blood flow play an important role in vessel growth or regression, and in the development of atherosclerosis (11,42). Bartels et al. showed high rates of SMC apoptosis in atherosclerotic lesions (42). Thus, loss of vascular smooth muscle cells (VSMC) by apoptosis would be expected to weaken the cap and predispose to rupture (11,39).

Apoptosis has been observed less consistently in primary atherosclerotic plaque than in restenotic specimens. It has been found more frequently in restenosis than primary human vascular lesions, because restenotic lesions are more proliferative than primary lesions (43).

Kovačević et al. in their study concluded that medical treatment which reduces inflammation and proteolysis in the aortic wall, and supports the VSMC recovery, is required to reduce the

aneurysm expansion and prevent rupture (38). Mallat et al. believe that strategies aimed at the inhibition of apoptosis may limit plaque erosion, thrombosis and progression (11).

### 1.2.2. Apoptosis in veins

SMCs, being a part of the local contractile units, are responsible for active venous tone maintenance (11,30,31). Autologous vein grafts are a common procedure for vascular reconstruction, but their patency rate is limited due to neointimal hyperplasia (NIH) that develops rapidly in veins subject to arterial blood pressure. The principal cause of graft failure is obliterative stenosis of the vessel because of proliferative thickening of the intima. A hallmark of neointimal lesions is SMC migration/proliferation and extracellular matrix deposition (44). Mayr et al. demonstrated that one of the initial events after grafting veins to arteries was SMC apoptosis followed by mononuclear cell infiltration and SMC proliferation (31,44-47).

Filis et al. evaluated a series of mediators regulating the apoptotic pathway and cell proliferation in human surgical specimens of varicose veins and healthy veins of the lower limbs. They showed that apoptotic deregulation is present in primary varicose veins. Bax, caspase 3, Bcl-xs and Ki-67 expressions were increased in great saphenous veins of patients suffering primary varicose veins. They could not detect Bcl-2 expression in their specimens (48). Increased apoptotic activity within the media of varicose veins has been shown by Bujan et al. The walls of healthy, control vein specimens acquired a more collagenous and papillomatous appearance with age (49).

In their study of apoptosis in primary varicose veins, Urbanek et al. documented an increase in SMC diameter, the presence of intracellular vacuoles, disruption of the fibers of the elastic network containing hypertrophic secretory SMCs, and extracellular matrix accumulation

(15,30,31,46). Simovart et al. found a trend of the number of apoptotic cells to increase in the walls of varicose veins along with the advancing age. The advancing age can itself be a factor that sensitizes cells to apoptosis (50,51).

### 1.3. Chronic renal insufficiency: etiology and pathogenesis

Acute kidney failure is a sudden loss of kidney function. Kidney damage maybe reversible if it is caused by serious injury from shock, trauma, an accident, medication, or obstruction.

Chronic renal disease is a progressive loss in renal function over a period of months or years. In the United States, the most recent data suggest that 27 million individuals have chronic kidney disease, representing nearly one in every seven adults and a 30% increase over the past decade. It rises dramatically with age, and is also associated with obesity and diabetes. Many patients with impending renal failure are regularly seeing their primary physician or a nephrologist. Symptoms may include: poor appetite, vomiting, bone pain, headache, insomnia, itching, dry skin, fatigue with light activity, muscle cramps, high urine output or no urine output, recurrent urinary tract infections, pale skin, bad breath, metallic taste in mouth, irritability, tissue swelling. Interventions, such as maintaining optimal blood pressure control, may delay or halt the development of progressive renal disease (52-57).

Therapy for the end-stage renal disease has evolved in the past 50 years so that three major treatments are available:1) hemodialysis, typically provided in a dialysis center but also performed at home; 2) automated peritoneal dialysis, usually chronic ambulatory peritoneal dialysis or chronic cycling peritoneal dialysis; and 3) renal transplantation from a living-related, living-unrelated, or cadaver donor (53,58).

### 1.4. Apoptosis and End-Stage Renal Disease

Apoptosis promotes the loss of renal epithelial cells that characterizes acute and chronic kidney diseases. Examples are podocytopenia and tubular cell loss in acute kidney injury and chronic tubular atrophy (3). Enhanced apoptosis is characteristic for chronic kidney disease (59).

End-Stage Renal Disease (ESRD) patients under hemodialysis (HD) are characterized by a chronic inflammatory state that includes aberrant and chronic production of inflammatory cytokines such as interleukin (IL)-6 (60). They are more likely to display spontaneous apoptosis (61,62).

It has been suggested that the systemic presence of uremic proteins and the dialysis procedure itself may promote immune cell activation and hence inflammation (60,63,64). The mononuclear cell recognizes the hemodialysis membrane as a foreign element and thus, in accordance with the immunologic concept, activates itself to produce a specific cellular response. These cells are more liable to die by apoptosis. This apoptosis is directly related to the degree of biocompatibility of the dialysis membrane (61,62). In hemodialysis patients, it is unclear whether increased apoptosis of neutrophils is due to uremia or HD itself. Previous studies have suggested that even though HD generates pro-apoptotic factors, the procedure causes only a transient sequestration of potentially apoptotic neutrophils (64,65).

### 1.5. Venous anatomy in the upper limb

Veins physiology is more complex than arterial physiology. Veins differ from arteries in a number of ways: have thinner walls, are collapsible, contain valves that are oriented to ensure unidirectional flow. Veins have a larger diameter than that of their accompanying arteries and in the periphery are usually duplicated (57,66-68).

- **Superficial veins**

The major superficial veins of the upper limb are the cephalic and basilic veins. As their name suggests, they are located within the subcutaneous tissue of the upper limb.

The basilic vein originates from the dorsal venous network of the hand. It ascends the medial aspect of the upper limb. At the border of the teres major, the vein moves deep into the arm. Here, it combines with the brachial veins to form the axillary vein (Figure 2) (69).

The cephalic vein arises from the dorsal venous network of the hand. It ascends the antero-lateral aspect of the upper limb, passing anteriorly at the elbow. At the shoulder, the cephalic vein travels between the deltoid and pectoralis major muscles (known as the deltopectoral groove), and enters the axilla region via the clavipectoral triangle. Within the axilla, the cephalic vein terminates by joining the axillary vein**.**

At the elbow, the cephalic and basilic veins are connected by the median cubital vein (66-69).

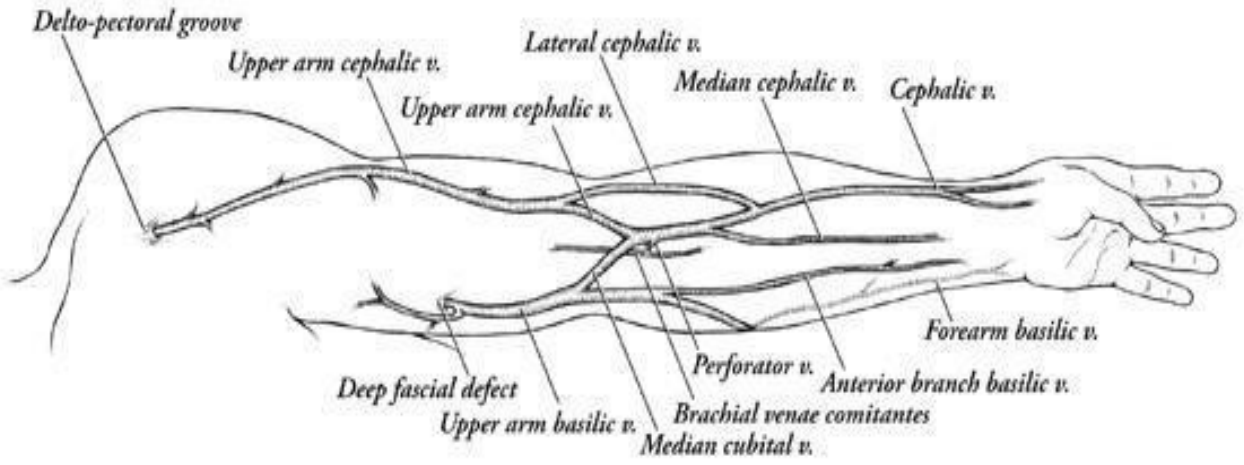



Figure 2. Venous anatomy in the upper limb adopted from Shenoy (69)

- **Deep veins**

The deep veins of the upper limb are situated underneath the deep fascia. They are paired veins that accompany and lie on either side of an artery. The brachial veins are the largest in size, and are situated on either side of the brachial artery. The pulsations of the brachial artery aid the venous return. Veins that are structured in this way are known as venae comitantes (67-73).

Perforating veins run between the deep and superficial veins of the upper limb, connecting the two systems. Lower extremity veins differ from upper extremity veins, but also deep conduit veins differ from superficial veins in terms of anatomy, histology and function.

Unlike calf veins, superficial forearm veins have an important role in thermoregulation (22,57,66,68,69,71,72,74,75).

### 1.6. Layers of the vein wall

The tunica intima is the inner layer of the vein and consists of a smooth, elastic endothelial lining. This surface has several different functions, one of which is its immunological properties that cause it to recognize foreign bodies within the vein. Injury to the endothelial lining can be mechanical, chemical or bacterial in nature.

The tunica media of the vein wall consists of muscle and elastic tissue. This layer is thick and comprises the bulk of the vein. The smooth muscle cells and the associated extracellular matrix is the major structural component of the vessel wall in both arteries and veins. They exist in the media of arteries and veins, bounded by the internal and external elastic laminae. The outer media is described as circular and the inner as longitudinal, reflecting the orientation of the cells (74). The nerve fibers that control vasoconstriction, vasodilation and that maintain muscle tone are also found in tunica media.

The adventitia is the outer layer of the vein and consists of connective tissue. It provides support and protection for the vein.

As the main cellular components of the blood vessels, ECs and SMCs play central roles in vascular biology and pathology (74,76).

### 1.7. Venipuncture

Venipuncture is the practice of obtained intravenous access. This can be for intravenous therapy, or obtaining a blood sample. The main location for venipuncture is the median cubital vein. This is a superficial vein that is situated anteriorly at the elbow. It is commonly used due to its accessible and superficial position. Intravenous sites on the extremities should be chosen from most distal to proximal. Venipuncture must be avoided in areas of flexion and extension (56,77-79). Puncture of a vein will leave a scar. When a fistula is created, such scars interfere with harmonious dilation and remodeling, cause turbulent flow, and predispose to stenosis (80-84). The veins of both arms, not only of the dominant arm, should remain untouched. It is important to instruct the patient in time and to motivate them that forearm veins are preserved (78,85). For venipuncture, the veins of the dorsum of the hand should be used as an alternative. Venipuncture complications may render veins potentially available for vascular access unsuitable for construction of a primary fistula (55,80,81). Because the patient who requires renal hemodialysis has usually undergone a period of intensive treatment requiring prolonged intravenous therapy, the readily available sites soon become consumed. Therefore, the selection of the site and type of shunt or fistula to be performed is equally important as the technique of performing the operation (55,56,57,77,80,81,82,86).

### 1.8. Hemodialysis

Hemodialysis is a life-saving treatment used to clean the blood of waste products and extra fluids. Chronic renal failure is a common illness with life-threatening manifestations that can be controlled with the use of hemodialysis (78,82,87).

The first clinical report of hemodialysis is credited to Wilhelm Kolph. Using hemodialysis, he managed a patient with acute renal failure in 1944. The dialysis was effective in controlling azotemia. His patient died after 12 hemodialysis treatments (53,57,66,88).

The number of patients with the End-Stage Renal Disease (ESRD) who require maintenance hemodialysis has risen sharply in the past two decades. It is approximately 300.000 in the United States and exceeds 25.000 in Turkey (89,90). Large clinical studies have shown that the mortality rate of patients undergoing HD is markedly elevated (60). These rates are striking: for example, a 30-year-old person with ESRD has the life expectancy of that of an 80-year-old person with the normal renal function (91).

Access to the blood contained within the vascular system was the dream of the ancient physician. Techniques of bleeding by incision, leeches and others were recognized as having therapeutic benefit and the concept of cleaning the blood and returning it to the body has endured since almost the beginning of recorded time. Access to the veins was obtained initially by the use of a quill and subsequently by various types of needles. These same methods were then applied to the arterial circulation. Repeated arterial and venous punctures, however, soon consumed the readily available sites. Initially, operations for access were used for a variety of disorders. These included blood dyscrasias, chemotherapy, and occasionally chronic hyperalimentation. With the development of specifically designed silastic catheters, these indications are now only rarely

valid, and these procedures are, in general, limited to the management of renal failure (53,57,58,63,92,93).

Since properly functioning vascular access is of the most importance to the patient with renal failure, the surgeon must consider many factors in the choice of operative procedures. These include the age and gender of the patient, the interval from access placement to planned use, and the anesthetic required to construct the chosen access. The decision to dialyze is often deferred until the situation becomes urgent. Each shunt or fistula operation must be planned carefully, as these patients with chronic renal failure will require a life-long therapy (53,58,88,94).

For emergency hemodialysis access, preferred are femoral vein catheters, which can be maintained in place for up to 48 hr. Femoral veins can be used repeatedly for several weeks. If a longer, albeit temporary, access is required, double-lumen catheters are used and placed percutaneously in the subclavian vein. This allows the patient to be ambulatory and the catheter has been well tolerated by the patients treated this way. The catheters are easily placed under local anesthetic and can and should be used immediately after placement (56,57,66).

### 1.8.1. Vascular access for hemodialysis

A vascular access is an access created by a connection between an artery and a vein, or connection between an artery and a vein with graft, whereby the vein or graft serves as an accessible conduit to remove and return blood during hemodialysis (8, NIH Publication No.14-4554 May 2014).

End-Stage Renal failure patients requiring a long-term hemodialysis need a durable vascular access (57,95,96).

More than 60% of all patients with ESRD who require chronic hemodialysis are accessed through an arteriovenous fistula (AVF) or arteriovenous graft (AVG), and the incidence is increasing at a rate of 2% to 4% per year. The goal of chronic vascular access is to provide repeated access to the circulation with minimal complications (88,89).

Good vascular access must be easy to prepare, long lasting, free from complications, and esthetically acceptable and economical (58,97).

With the increasing number of patients sustained on chronic hemodialysis, access related surgery forms a significant portion of vascular surgical practice (95). Performance of a successful hemodialysis procedure requires a functional vascular access (98,99). Ferrari et al. defined vascular access for dialysis as the "Achilles heel", but also the "Cinderella" of dialysis, indicating the poor consideration of the problem whether in the surgical environment, or in incomprehensible way in that nephrologic (97,100).

Arteriovenous fistulas require fewer interventions than grafts to maintain long-term patency for dialysis. For this reason, the National Kidney Foundation Dialysis Outcomes Quality Initiative guidelines recommend placement of fistulas in preference to grafts whenever the vascular anatomy permits it (96,99,101,102).

The three principal forms of chronic vascular access for hemodialysis are native arteriovenous fistulas, arteriovenous synthetic grafts, and double-lumen tunneled cuffed catheters. The first two types are conduits between the artery and vein, which provide a rapid blood-flow rate (103). Of these, the AVF comes the closest to being an ideal long-term hemodialysis vascular access (55,58,80,101).

The management of the vascular access must preview one tight collaboration between nephrologists, nurses, patient, vascular surgeon, and radiologist (100).

#### 1.8.1.1. Acute hemodialysis access

The usage of temporary and permanent dialysis catheters for hemodialysis vascular access has been on continual increase (93). In some patients, the only access for hemodialysis is possible through a central vein catheter. The first catheter was introduced by Sheldon in 1961 (96).

- **Percutaneous non-tunneled catheters** intended for temporary access
- **Double-lumen tunneled cuffed catheters (tunneled central venous catheter)** intended for prolonged usage

Cuffed *versus* uncuffed is indicated whether they are equipped with a subcutaneous cuff to promote tissue ingrowth and further fixation of the catheter (85). Use of an uncuffed catheter for periods of time beyond several weeks results in a relatively high rate of infection and is not recommended. Dacron or felt cuffs bonded to the catheter reduce the incidence of line-related infection and of catheter migration and must be used whenever a longer-term use of the catheter is anticipated, or when it is anticipated that a patient will be discharged from the hospital with a catheter remaining in place (53,79,96).

The optimal insertion side is the right internal jugular vein. The subclavian site should generally be avoided because it is associated with a higher incidence of insertion-related complications. Catheterization of the femoral vein is a good choice when the need for hemodialysis is expected to be short (<1 week). It is useful for performing the initial hemodialysis treatment in patients who present with acute pulmonary edema, because the patient head and chest can be elevated during insertion (66,79,92).

All catheters need to be inserted into hospitalized, bed-ridden patients.

In recent years, the use of tunneled central venous catheters (CVC) has grown exponentially. But, their use is not always justified. In comparison with grafts, CVCs have various

disadvantages including insertion-related complications, possible malfunctioning, risk of infections and thrombosis, but above all a high risk of steno-occlusion of central veins (104,105). These should be avoided at all costs, except as a temporary measure or when the life expectancy of the patient is short. Unfortunately, this often is difficult to accomplish at a clinical level because this is the most convenient way to obtain immediate dialysis access (53,98). Cifarelli et al. in their experience consider some conditions in which the use of the CVC is a priority, namely in patients with severe cardiopathy and reduced ejection fraction in whom volume overload caused by a graft access increases the risk of cardiac failure; in pediatric patients weighing less than 20 kg, in whom a graft vascular access could cause notable, even insuperable difficulties of construction and management, as well as negative psychological implications for the small patient; in very elderly patients in poor clinical condition with a short life expectancy or suffering from cancer, in whom a CVC could be used also for the infusion of chemotherapeutic drugs; and in patients with peripheral arteriopathy in whom the alternative is a graft in a lower limb, because of the high risk of ischemia (88,93,99,104).

Complications that occur at the time of catheter insertion are secondary to inexperience, pneumothorax, arterial/venous injury, brachial plexus/phrenic nerve injury, cardiopulmonary complications, mechanical failure, thrombosis and infection. Clearly, these issues can be avoided when the fistula has been constructed in a timely fashion in pre-dialysis patients, so that it is ready for use prior to the need for maintenance dialysis (53,55,57,66,78,105).

Whether arguing the benefits of “Fistula First” or "Catheter Last," the fact that clinicians are in need of an alternative to expanded polytetrafluoroethylene (ePTFE) is irrefutable. While the bulk of this effort has historically focused on developing new synthetic biomaterials, more recently,

investigators have developed a variety of cell-based strategies to create tissue-engineered vascular grafts (82,99,106,107).

Every patient should undergo chest radiography after a central catheter is placed.

#### 1.8.1.2. Chronic hemodialysis access

##### 1.8.1.2.1. Arteriovenous fistulas

In 1966, Brescia and colleagues described a technique for creating an arteriovenous fistula based upon the radial artery and any available forearm vein. Even today, forty years later, "the Cimino-Brescia radiocephalic fistula... remains unquestionably the best available form of vascular access for hemodialysis because of its unequaled long-term patency rate and minimal complications" (84,96,105,108).

The groundbreaking article by Brescia and Cimino in 1966 revolutionized the creation of the vascular access, and the Cimino fistula was soon used in almost all dialysis patients (55,80,96,109). The surgeon usually places an AV fistula in the forearm or upper arm (Figure 3) (69). An arteriovenous fistula is the most preferred form as it is essentially free of infection, provides a high blood flowrate, lasts longer than other types of access and is the least likely to clot (53,55,78,84,101,105).

Routine preoperative sonographic vascular mapping is recommended before each access surgery to assist the surgeon in determining the optimal location for fistula creation (55,58,71,72,78, 102).

Although several techniques for anastomosis are available, the side-to-end anastomosis has deservedly become the most commonly used technique. It is absolutely indicated when artery and vein are far apart and must be brought closely together to create an anastomosis. As a further

advantage, a venous thrombosis will affect only the venous limb if it supervenes. If the fistula has to be revised, it is easy to create an anastomosis at a more proximal site. Because the number of vascular access sites is limited, the preservation of each site for as long as possible is important for the long-term management of these patients (55,78,80,84,88,96,105).

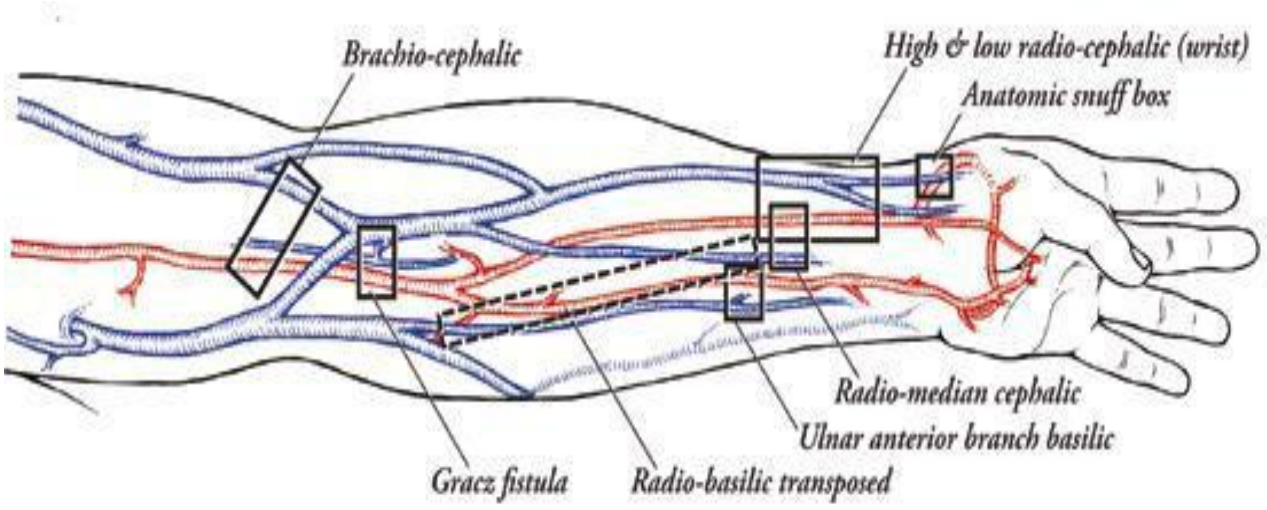



Figure 3. Primary sites of AVF creation adopted from Shenoy (69)

The anatomical snuffbox fistula may be the first considered in any patient requiring hemodialysis access, because failure still leaves several options for fistula available on that limb (110).

It is well recognized that AVFs, as access for hemodialysis, are less prevalent among patients of older age, of female gender, with obesity, diabetes or cardiovascular disease (86,96,111).

Patients who benefit from preoperative ultrasound evaluation are those with:

- insufficient clinical examination (obese, absent pulses, multiple previous access surgery)
- possible arterial disease (older age, diabetes, cardiovascular disease)
- possible venous disease (previous cannulation) (71,72,73,90,94,111,112).

The biology of the AV fistula is fascinating and the adequate management of the fistula and its complications should be based on pathophysiologic reasoning (80,109). Complications of AV fistula include thrombosis, infection, bleeding, increased venous pressure, arterial insufficiency, aneurysm, carpal tunnel syndrome, distal ischemia, and even heart failure. Complications related to AV fistula increase with age, because of comorbidities (53,55,73,80,83,94).

- **Maturation of fistula**

"Maturation" of an AVF involves dilation of the runoff vein, thickening of its wall, and eventually enlargement of the afferent artery. Therefore, the quality and diameter of the artery plays a major role in the maturation of the AVF (53,57,69).

Banerjee et al. referred to a "rule of sixes" in the fistula assessment. At 6 weeks post-creation, the diameter of the body of the fistula should be at least 6mm. The depth should not be more than 0.5 to 0.6cm. The blood flow rate should be 600ml/min or more by this time. The length of the fistula should be 5 to 6cm to allow for a successful two-needle dialysis (103).

Delay in maturation or failure of maturation of Cimino-Brescia fistulas contributes to the significant vascular access-related morbidity of chronic hemodialysis patients (109,114).

An AV fistula frequently requires 2 to 3 months to develop or mature, before the patient can use it for hemodialysis. A significant number of AVF (28-53%) never mature to support dialysis. Often, nephrologists and vascular surgeons wait up for 6 months and even longer hoping that the AVF will eventually grow to support dialysis. In general, a blood flow of 500ml/min and a diameter of at least 4mm are needed for an AVF to be adequate to support dialysis therapy (101,115,116,117).

The first step in a systematic evaluation of the mature AVF is to examine the integrity of the skin overlying the fistula, which should appear normal without erythema, focal masses, or focal swelling (53,82,110,118).

#### 1.8.1.2.2. Arteriovenous graft

For more than 30 years, research and industry have attempted to introduce into clinical practice solutions and products that could remedy the impossibility to use native veins. AV grafts are reserved for patients whose vascular anatomy does not permit construction of a native AV fistula (55,104,107).

Vascular grafts of various types have been created that would approach the ideal characteristics as closely as possible those are a low antigenic power, high resistance to infections, low risk of thrombosis, and easy pierceability but high resistance to puncturing (90,104).

The prostheses nowadays used in the vascular access for hemodialysis have low patency rates, due to the luminal obstruction, determined by the intimal hyperplasia (99). An AV graft is more likely than an AV fistula to have problems with infection and clotting. Repeated blood clots can block the flow of blood through the graft (55,95). 11% to 35% of patients develop infection, which represents one of the main causes of access failure (119). In view of their complication profile, the native vein arteriovenous fistula should continue to be the first choice for vascular access among patients requiring chronic hemodialysis (56,77,95,99).

- **Maturation of graft**

Although some advocate immediate use of an AV graft for hemodialysis, adhesion between the graft and the subcutaneous tunnel to prevent hematoma formation requires at least 2-3 weeks. Graft is considered mature when edema and erythema have resolved and the graft can be easily

palpated. The graft should be cannulated immediately postoperative only to avoid risks from temporary catheters (81,97,99,105).

### 1.8.2. How does a patient care for and protect vascular access?

A patient can care for and protect a vascular access by:

1. Ensuring that the health care provider checks the access for signs of infection or problems with blood flow before each hemodialysis treatment, even if the patient is inserting the needles.
2. Keeping the access clean at all times.
3. Using the access site only for dialysis.
4. Being careful not to bump or cut the access.
5. Checking the thrill in the access every day. The thrill is a rhythmic vibration a person can feel over the vascular access.
6. Watching for and reporting signs of infection, including redness, tenderness, or puss.
7. Not letting anyone put a blood pressure cuff on the access arm.
8. Not wearing jewelry or tight clothes over access site.
9. Not sleeping with the access arm under the head or body.
10. Not lifting heavy objects or putting pressure on the access arm (82,85,116,120).

### 1.8.3. Failure of arteriovenous fistula

Dysfunction of the AVF is a common problem in hemodialysis patients and remains a major cause of morbidity and hospitalization (55,80,119,121). The failure of an AVF may be defined as the early or late failure:

1. the early failure: inflow and outflow problems;
2. the late failure: venous stenosis, arterial stenosis and thrombosis.

The early fistula failure or a failure to mature fistula is defined as a fistula that has never matured to be useful, is difficult to cannulate, or fails to generate necessary blood flow for a successful two needle dialysis (103).

Miller et al. showed a higher primary nonfunction rate in forearm fistulas as compared with upper arm fistulas (59% *versus* 34%) (119).

The 1-year patency rates of AVF are estimated to be 63%, and recurrent AVF failure is a major cause for morbidity and mortality of hemodialysis patients (55,78,80,82,121). Studies relating to preoperative venous mapping for AVF construction underwent systematic review. Doppler ultrasound is the preferred method for preoperative vascular mapping. Preoperative vascular mapping was shown to substantially increase the total proportion of patients dialyzing with fistulas (53,55,71,72,82,101,107,121,122).

The pathogenesis of the early native arteriovenous fistula failure (juxta-anastomotic stenosis) is complex and multifactorial. Causative factors include a small artery (<1.5 to 2 mm) and a small vein (<2.0 to 2.5 mm), surgical manipulation and less-than-ideal technique, previous venipunctures, the development of accessory veins that direct blood away from the primary venous drainage channel, hemodynamic stressors, and a possible genetic predisposition to vasoconstriction and neointimal hyperplasia after endothelial and smooth muscle injury (107,109,119,121,122,123). Roy-Chaudhury et al. in 2006 recognized that all vascular manipulations (surgery or balloon angioplasty) cause endothelial and smooth muscle cell injury, which results in a restenotic process. Therefore, these interventions need to be linked to therapies

that can target both the traditional and the alternative pathways that are involved in the pathogenesis of neointimal hyperplasia and vascular stenosis (55,117,119,123).

The possible biologic reasons for an AVF to undergo maturation failure are as follows:

- Failure of the arterial dilation because AVFs are created in patients with severe vascular disease and diabetes;
- Failure of the venous dilation because of the aggressive push toward trying to create a native AVF whenever possible and it could result in the use of a poor venous segment that has lost the ability to vasodilate because of previous venipuncture (80);
- Significant stenosis at the anastomosis, which is the most common anatomic abnormality in fistulas that fail to mature (121);
- Accelerated venous neointimal hyperplasia as a result of vascular injury of the segment of vein that has been mobilized and manipulated by the surgeon during the procedure. This process often involves stretching, torsion and skeletonization of the vessel, which may disrupt the *vasa vasorum* for that segment of the vein. Morphologically, neointimal hyperplasia results from proliferation of SMCs combined with matrix deposition (54,115).

The chronic kidney disease is associated with vascular damage, and it is likely that uremia may impair AVF patency, too (54).

To preserve the vascular system, it is important to avoid blood withdrawals or intravenous infusions from the arm and forearm, and to use the veins of the hands for these purposes (108).

The four main problems identified as associated with occlusion are: (1) low blood pressure during hemodialysis; (2) successive fistula puncture sites located too close to one another; (3) abnormal blood flow; and (4) poor moisture control.

Age is an additional independent factor associated with the prevalence of fistulas. Other factors include presence of peripheral vascular disease, obesity and lower socioeconomic status. Allon et al. showed that female gender and black race are independent predictors of a lower likelihood of fistula placement (55,85,117,123).

## 2. HYPOTHESIS

Apoptosis correlates positively with previous venipuncture and failure of AVF.

## 3. AIMS OF STUDY

### 3.1. GENERAL AIM:

To investigate the impact of apoptosis in the native vein wall used for arteriovenous fistulas for hemodialysis on the failure of fistulas.

### 3.2. SPECIFIC AIMS:

1. To evaluate apoptosis in the native vein wall of patients who will undergo a surgery for AVF as dialysis access.
2. To compare apoptosis between the veins previously punctured or not.
3. To assess the number of patients with AVF failure and correlate it with apoptosis.

## 4. MATERIALS AND METHODS

### 4.1. Subjects

Vein specimens were obtained from 60 patients with terminal chronic renal disease, at the Clinic of Vascular Surgery in University Clinical Center of Kosovo, who, for first time, underwent a surgery for AVF placement as dialysis access. The indication for creation of a vascular access was done by a nephrologist, and his staff advised the patient on how long to fast prior to the procedure, and which medications to take or not to take.

Patients in whom AVF did not develop to sustain dialysis or was thrombosed before the first successful cannulation for hemodialysis, as well patients in whom AVF was made with graft prosthesis, were excluded from the study.

**Group I (study group)** consisted of 30 patients in whom the vein which was used for AVF placement had been previously punctured (for blood draws or for intravenous lines), irrespective of the purpose of puncture.

**Group II (control group)** consisted of 30 patients in whom the vein which was used for AVF placement had not been previously punctured.

The patients were prepared for surgery, provided their systolic blood pressure would not be less than 100 mmHg. Both upper extremities were then examined, and the extremity which had an appropriate artery and vein, at preferably the nondominant extremity, was chosen for the placement of the fistula. The most distal possible site was chosen for AVF placement.

All patients had a competent deep vein system without a history of thrombotic episodes.

For each patient, the following data were included for analysis: data related to age, gender, time of AVF surgery, duration of renal failure, duration on dialysis or the placement of fistula made for preventive purposes, site of fistula, type of fistula, Doppler sonographic data before fistula

placement, presence of comorbid conditions, such as diabetes mellitus, arterial hypertension, pulmonary disease, previous cerebrovascular disease, previous peripheral vascular disease, rheumatologic disease, cancer, use of ACE inhibitors, statins, calcium antagonist, coumarin, platelet aggregation inhibitor, prior central catheter placement, HBs antigen, HCV, HIV, history of intravenous drug abuse, and smoking.

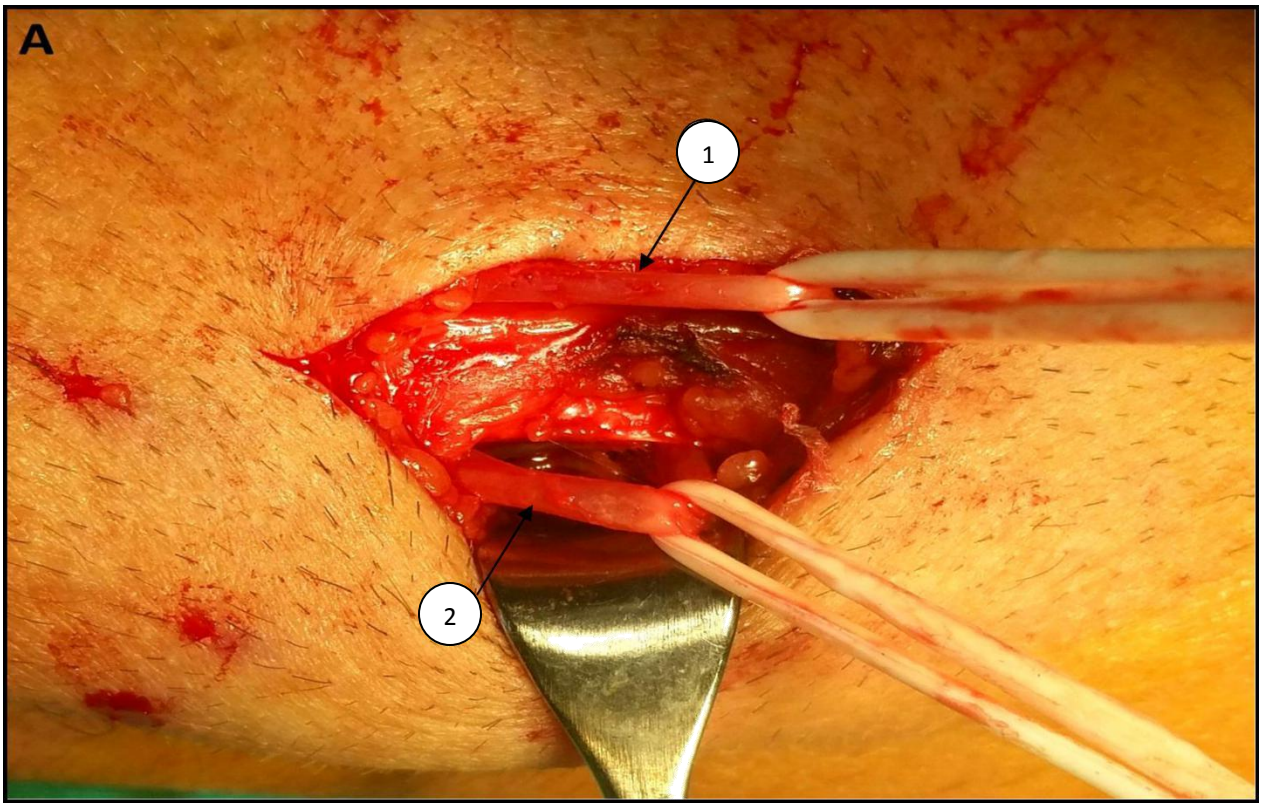
All preoperative sonographic mapping studies were performed by a vascular surgeon. A Siemens Acuson X300 system with a 7- to 15-MHz transducer was used. The patient was placed in the supine position with the arm to be examined comfortably extended at approximately 60 degree angle from the chest. The veins were sequentially evaluated along the arm for diameter, patency, and depth. Stenosis and thrombosis of the draining or central vein were criteria for exclusion from the study.

The conditions of the artery and vein were examined by the surgeon and based on the degree of arterial atherosclerosis, blood flow and the presence of thrombosis, they were divided into three types: excellent, good and sufficient, as follows:

- **Excellent artery**: a minimum of 2 mm in internal diameter, a soft and elastic artery wall with no atheroma and a good flow;
- **Good artery**: a minimum of 2 mm in internal diameter, a relatively stiff artery wall but with no atheroma and an average to good flow;
- **Sufficient artery**: a minimum of 2 mm in internal diameter, a hard and fragile artery wall with atheroma and an average flow;
- **Excellent vein**: a minimum of 2 mm in internal diameter, a normal wall, lacking any clots and thrombosis, completely open proximal, a good flow, and dilates and fills well with pressure on the proximal part of the vein;

- **Good vein**: a minimum of 2 mm in internal diameter, an appropriate wall, lacking proximal obstruction, but having proximal stenosis, which could be removed by a dilator. The vein will appropriately fill up with blood after release of the proximal pressure;
- **Sufficient vein**: a minimum of 2 mm in internal diameter, a stiff wall, relative stenosis or obstruction of the proximal, in which case a dilator of maximum size-2 will pass through. A low flow rate and when controlling the proximal part of the vein, does not fill up appropriately with blood and will not dilate desirably.

To all patients vascular access was performed on an outpatient basis, under local anesthesia, in one or other arm. Radial artery-cephalic vein forearm fistula or brachial artery-cephalic vein upper arm fistula were placed, with termino-lateral anastomosis. The vein taken during the procedure was always the cephalic vein, and it was 1cm longer than usually taken during the operation. Before placement of AVF, 1-cm vein segment was excised from the distal part of the vein for immunohistochemical analysis. Then, the vessel was irrigated and dilated with heparin-saline solution. Afterward, we performed end-to-side arterio-venous anastomosis using 6/0 or 7/0 polypropylene suture material (Figure 4).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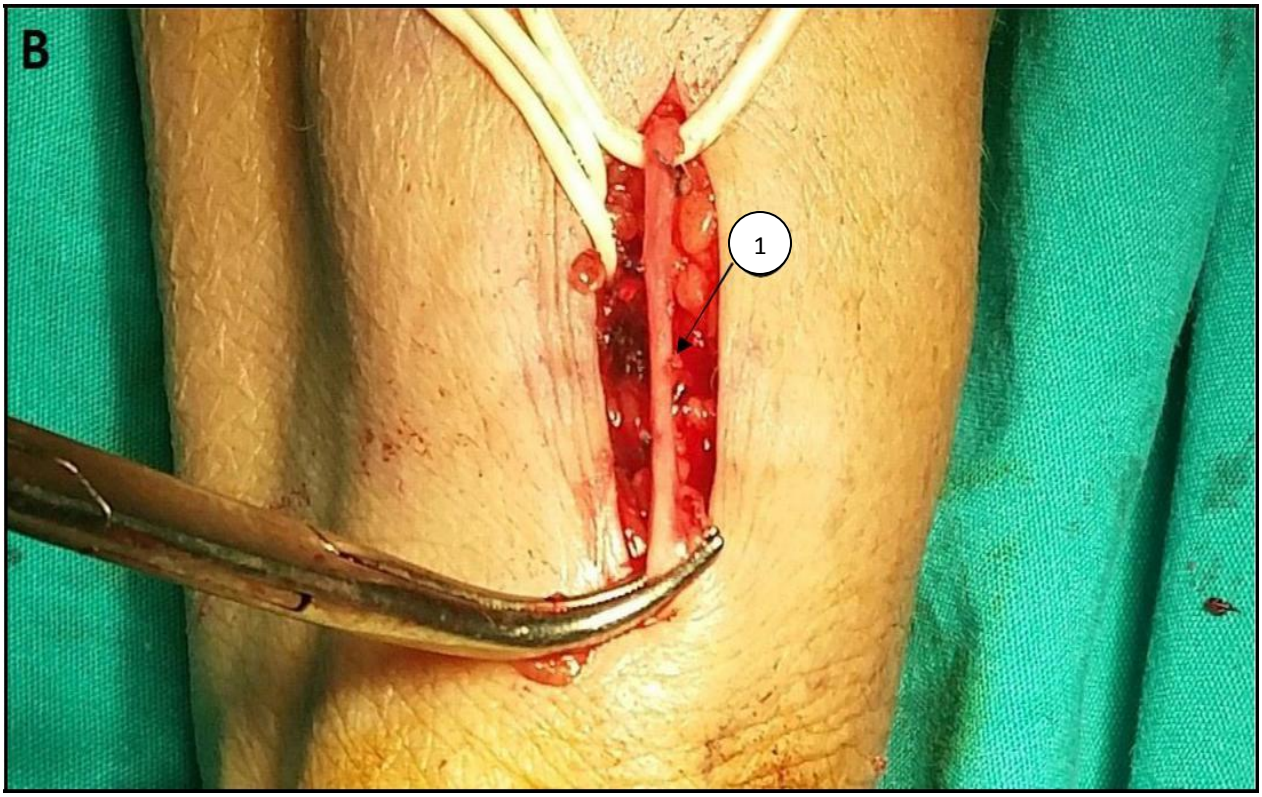
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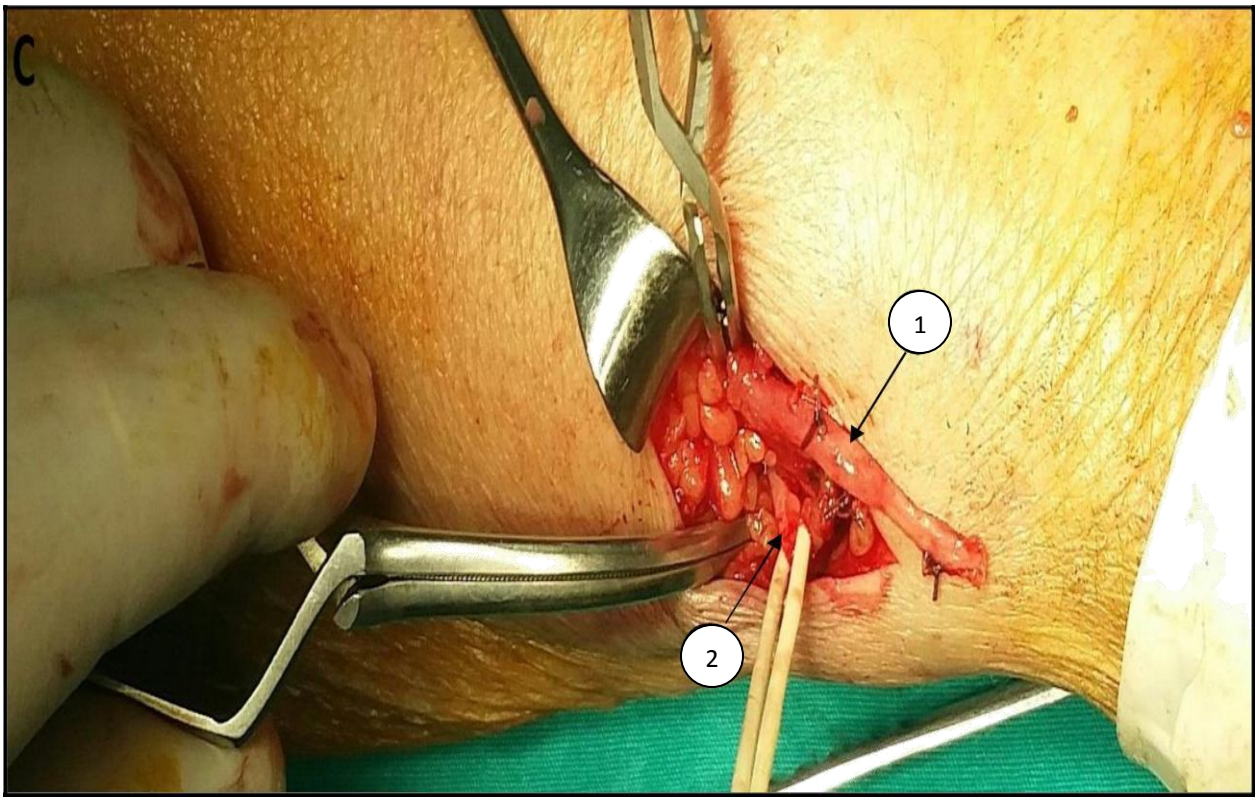
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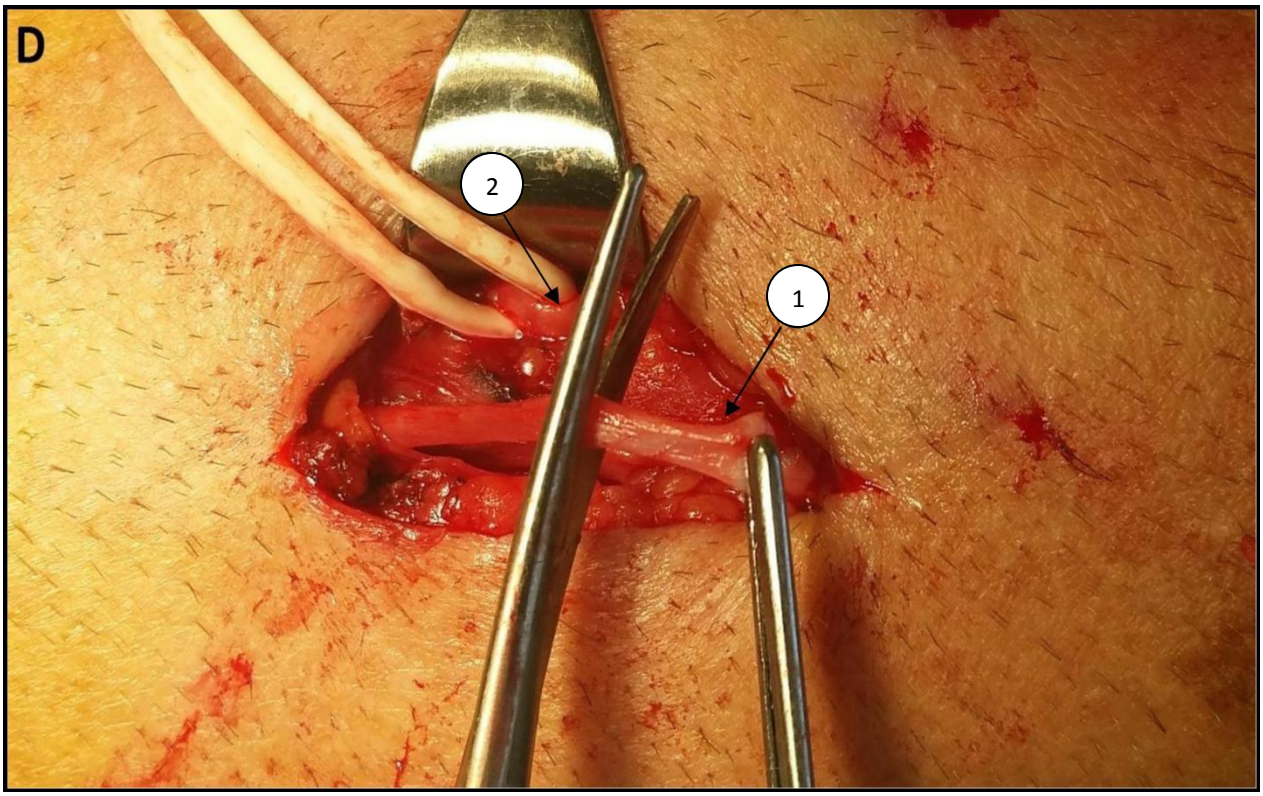
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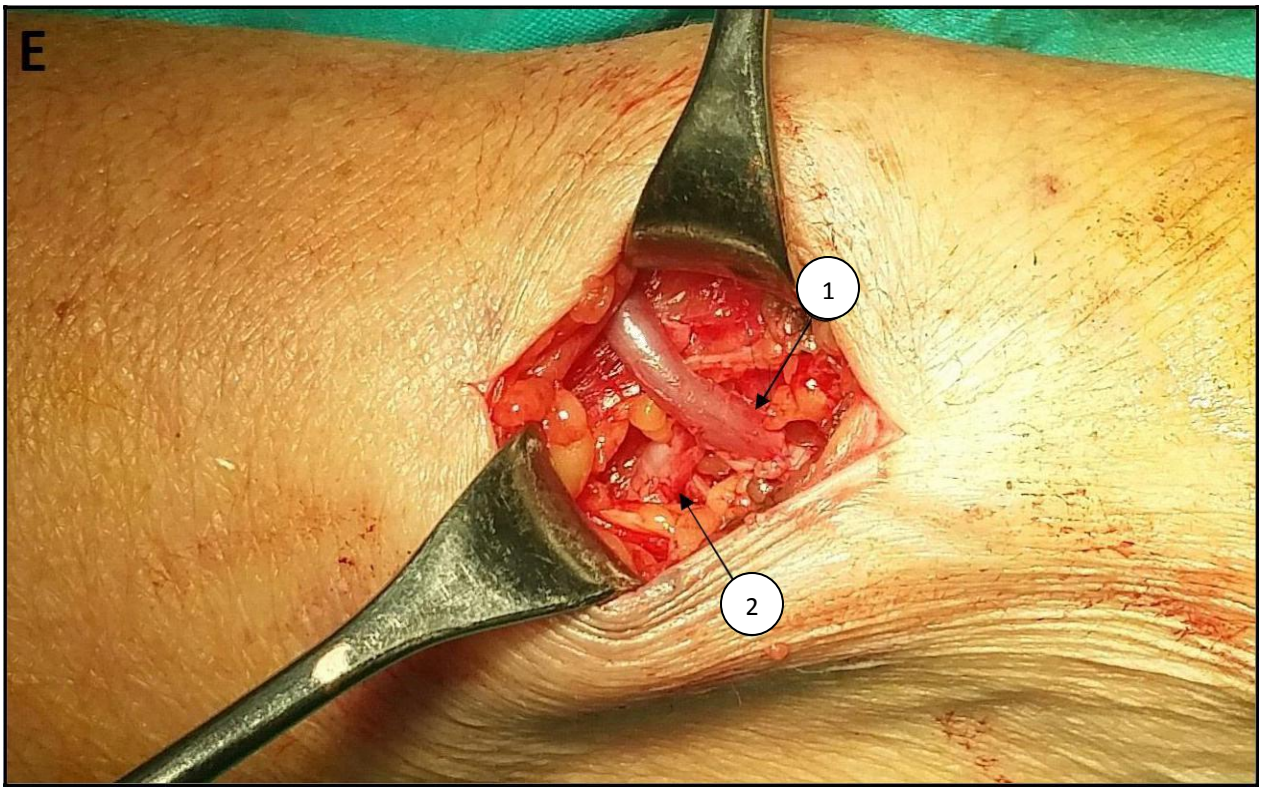


Figure 4. Procedures for retrieving vein material. A. Preparation of artery and vein. B. C. Vein division. D. Retrieving the venous segment (1cm). E. Radial-cephalic fistula;
Note: 1. Cephalic vein, 2. Radial artery.

The fistula maturation took 4-6 weeks or depending on the patient conditions. Until then, patients did the dialysis in the central venous catheter, except when AVF was done for preventive purposes.

Patients were followed on an outpatient basis after 3, 6, 9 and 12 months and the failure of AVF was noted. All patients were dialysed on standard bicarbonate basis for 4h three times weekly using biocompatible polysulphone HD membranes (Fresenius).

### 4.2. Tissue preparation

All vein specimens were divided into two portions along longitudinal axis. The specimens collected were fixed in 10% buffered solution containing approximately 4% formaldehyde for

24h and embedded in paraffin for conventional histology and immunohistochemistry. Tissue preparation took place at the Department of Pathology of the University Clinical Center of Kosovo, in Prishtina.

Histology and immunohistochemical analysis were made in "Ljudevit Jurak" Department of Pathology, University Hospital Center "Sisters of Mercy", Zagreb, in my presence.

### 4.3. Histology

Specimens were stained with hematoxylin and eosin for routine histological evaluation. Results were evaluated by two independent investigators who were blinded to the patient's clinical findings. All vein specimens were stained with Mallory trichrome, Gomori's and von Gieson's histochemical staining to assess the pathological changes in the amount of collagen connective fibers and to differentiate between collagen and smooth muscle fiber as well as the elastin network. Histology was performed for confirmation that the sections used for immunohistochemical analysis contained the classical findings of veins which were previously punctured and those which were not previously punctured.

### 4.4. Immunohistochemistry

Routinely, identification of smooth muscle cells was performed by experienced histopathologist using morphological criteria.

For immunohistochemical analysis, the following primary antibodies were used overnight at 4 °C:

- p53 mouse monoclonal antibody (DAKO), dilution 1:50;
- Bcl-2 mouse monoclonal antibody (DAKO), dilution 1:50;

- Caspase 3 mouse monoclonal antibody (Abcam), dilution 1:50;
- Bax rabbit polyclonal antibody (DAKO), dilution 1:500.

For indirect streptavidin–biotin–peroxidase method, instructions from the manufacturer (DAKO) were followed. Biotin-conjugated secondary antibody was applied in a 1:200 dilution for 1 h at room temperature, then a 30-min incubation in Strept–AB complex was done, and for color development 3,3′-diaminobenzidine tetrahydrochloride and hematoxylin as a counterstain were used.

Qualitative and quantitative evaluation of the immunohistochemical staining was done using a computer with image analysis software according to the article by: Filis et al. (48). The expression of proteins was evaluated according to a semiquantitative assessment of positive cells. Scoring was performed as: no staining, minimal staining (1-3%), moderate staining (>3-50%), and maximal staining (>50-100%). Cells that stained positive (cytoplasmic and nuclear staining) for the examined antibodies in the intima, media, and adventitia were counted at 400× magnification and quantified in 10 random fields per section (16).

As positive controls for Bcl-2, caspase 3 and Bax, tonsillar tissues were used, and for p53, breast tissues. Negative control was obtained by omitting primary antibody.

### 4.5. Statistical analysis

Data management and analysis: Chi square test or Fisher Freeman Halton test were used to test the differences in proportions of qualitative variables between groups. Normality of quantitative variable distributions was tested by Kolmogorov-Smirnov test. Mann Whitney U test was used for testing the differences between quantitative variables which did not follow normal distribution. The level $p<0.05$ was considered as the cut-off value for significance. A descriptive analysis is presented in tables and figures.

### 4.6. Ethics

The study was conducted according to all currently valid and applied guidelines whose purpose is to assure proper conduction and protection of persons included in this research as examinees.

Ethical approval for the study was obtained from University Clinical Center of Kosovo, in Prishtina, University Hospital Center “Sisters of Mercy” in Zagreb, and University of Zagreb, School of Medicine, Zagreb.

Identity of patients remained confidential and protected.

A freely given, written informed consent was obtained from participants or from witnesses.

## 5. RESULTS

### 5.1. Clinical characteristics of patients

According to the preoperative physical examination as well as noninvasive imaging findings, 60 patients on maintenance HD with an AVF as vascular access were found to be appropriate candidates for this study. There were 30 patients (18 men, 12 women; median age 63.50 years; IQR=23.00 years) in group I (study group), and 30 patients (20 men, 10 women; median age 63.00 years; IQR=20.25 years) in group 2 (control group). Co-morbid conditions were seen in all patients, and included hypertension (26/21) in both groups, hematologic disease (19/17), and diabetes (11/11) (Table 1).

There were no statistically significant differences among the patients in the study and control group in age, gender, dialysis performed, central venous catheter insertion, HBsAg, HCV, HIV, and comorbidities (hypertension, pulmonary disease, hematologic disease, diabetes, peripheral vascular disease, cerebrovascular disease, rheumatologic disease, cancer) (Table 1).

Table 1. Characteristics of patients (N=60)

| | **Group I** study group (N=30) | **Group II** control group (N=30) | **p Value** |
|---|---|---|---|
| **Age (years) Median/IQR** | 63.50 | 63.00 | 0.695** |
| **Gender (male/female)** | 18/12 | 20/10 | 0.592* |
| **No dialysis** | 11 | 10 | 0.787* |
| **Central venous catheter** | | | 0.713* |
| - Internal jugular vein | 6 | 9 | |
| - Femoral vein | 11 | 8 | |
| - Subclavian vein | 2 | 3 | |
| HBsAg positive | 2 | 2 | 1.000* |
| HCV positive | 1 | 2 | 1.000* |
| HIV positive | 0 | 0 | |
| **Comorbidities** | | | |
| - Hypertension | 26 | 21 | 0.117* |
| - Pulmonary disease | 3 | 3 | 1.000* |
| - Hematologic disease | 19 | 17 | 0.598* |
| - Diabetes | 11 | 11 | 1.000* |
| - Peripheral vascular disease | 2 | 2 | 1.000* |
| - Cerebrovascular disease | 4 | 0 | 0.112* |
| - Rheumatologic disease | 2 | 1 | 1.000* |
| - Cancer | 2 | 5 | 0.424* |

***-**Fischer's Exact Test, ****-**Mann-Whitney U Test

The greatest number of fistulas was observed in the non-dominant extremity, 52 fistulas (86.7%) of the total number of 60, the finding in line with the data from literature. There were 35 (58.33%) radiocephalic fistulas and 25 (41.67%) brachiocephalic fistulas, with no significant difference between the two groups (Table 2, Figure 5).

Table 2. Intraoperative characteristics (N=60)

| | **Group I** study group (N=30) | **Group II** control group (N=30) | **p Value** |
|---|---|---|---|
| **Choice of access site** | | | 0.254 |
| - non dominant extremity | 24 | 28 | |
| - dominant extremity | 6 | 2 | |
| **Type of fistula** | | | 0.892 |
| - R/C sin***** | 15 | 15 | |
| - B/C sin***** | 10 | 12 | |
| - R/C dex***** | 3 | 2 | |
| - B/C dex***** | 2 | 1 | |
| **Condition of artery** | | | 0.689 |
| - sufficient | 1 | 2 | |
| - good | 16 | 18 | |
| - excellent | 13 | 10 | |
| **Condition of vein** | | | 1.000 |
| - sufficient | 3 | 3 | |
| - good | 13 | 13 | |
| - excellent | 14 | 14 | |

*- R/C-radiocephalic fistula, B/C- brachiocephalic fistula, p-Fischer's Exact Test

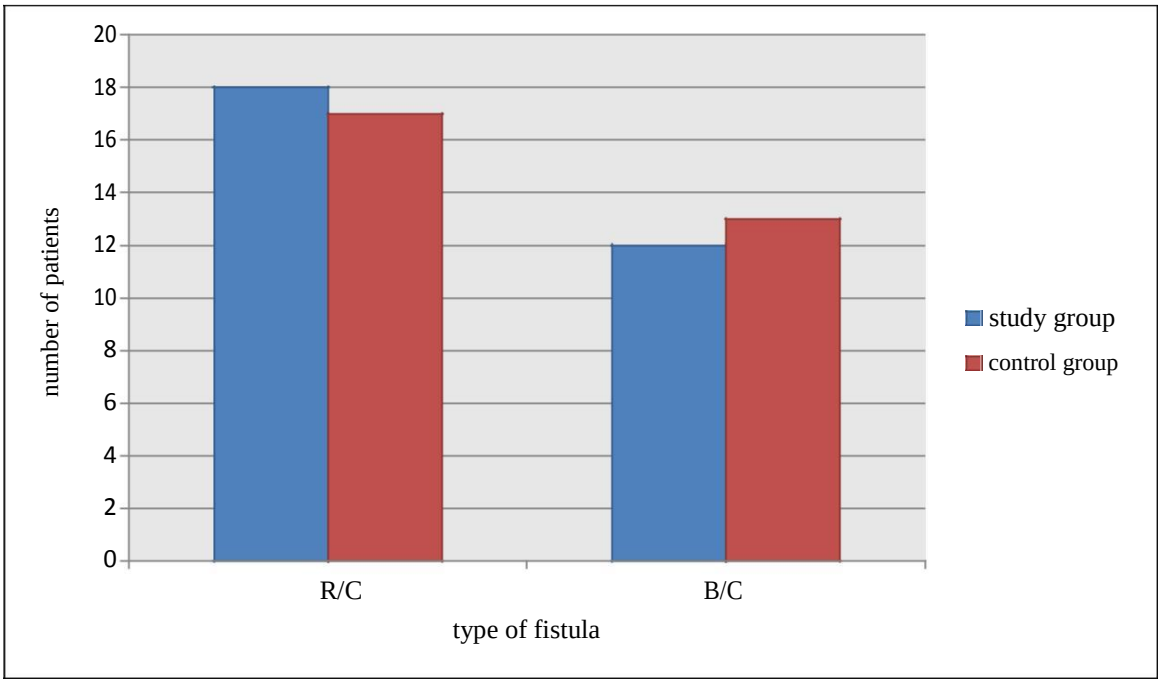


Figure 5. Type of fistula performed in both groups.
Note. R/C-radiocephalic; B/C-brachiocephalic

### 5.2. Histology

Routine hematoxylin and eosin staining showed histological appearance of the specimens of nonpunctured vein (control vein) (Figures 6 and 7), and punctured vein (study vein) (Figure 8).

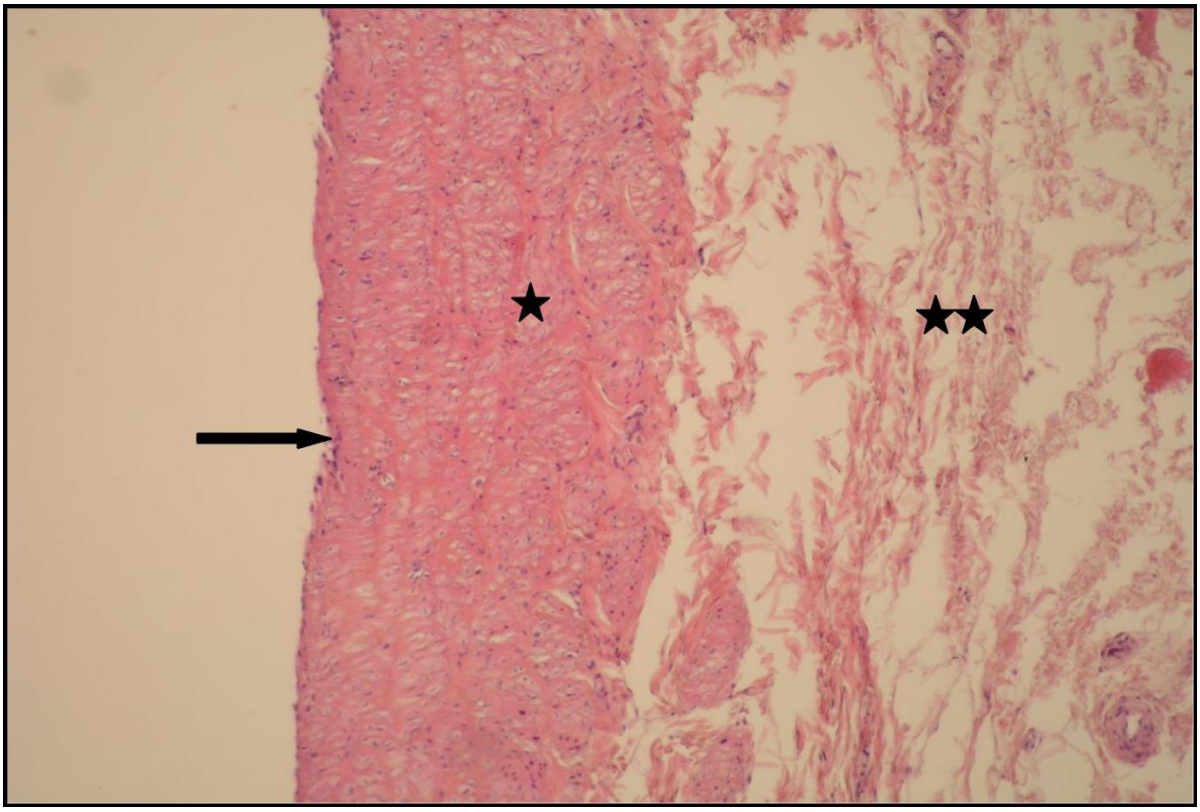

Figure 6. The control vein. The intima (endothelium) (arrow); media (one star); adventitia (two stars). HE, x100.

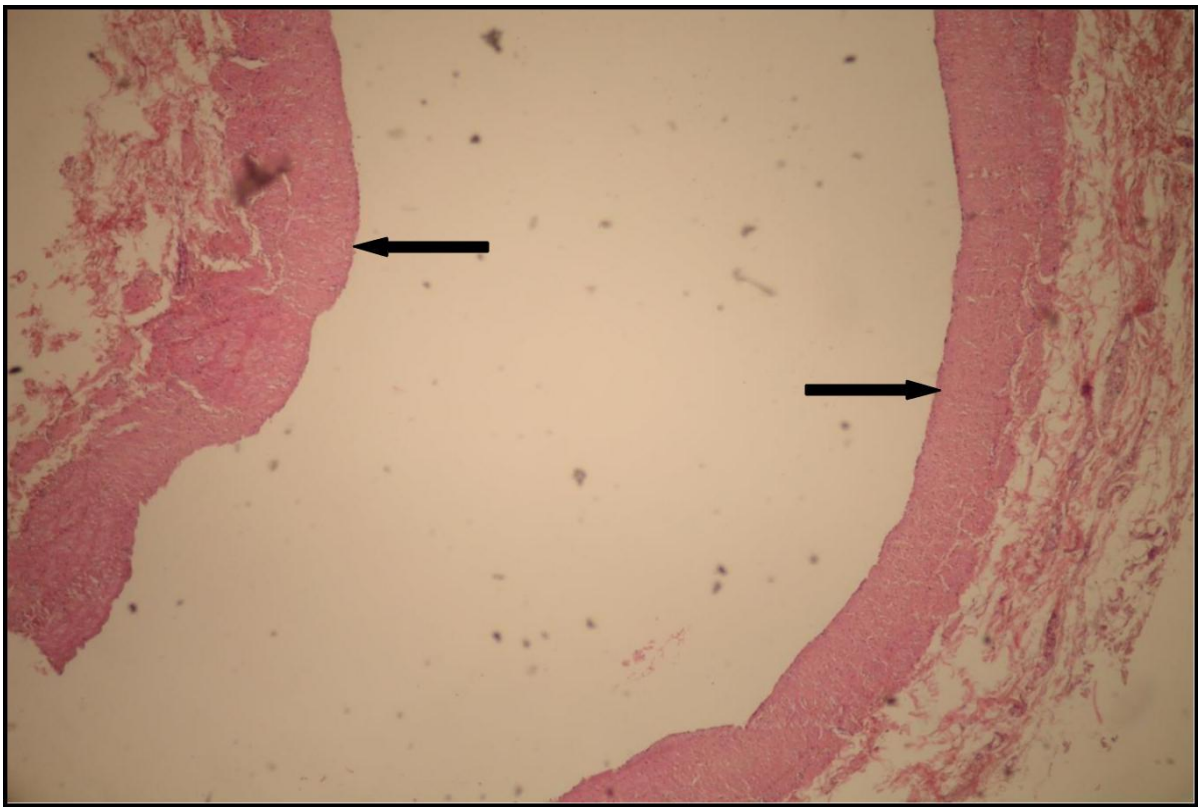

Figure 7. The control vein. The arrow points to the intima of vein. HE, x40.

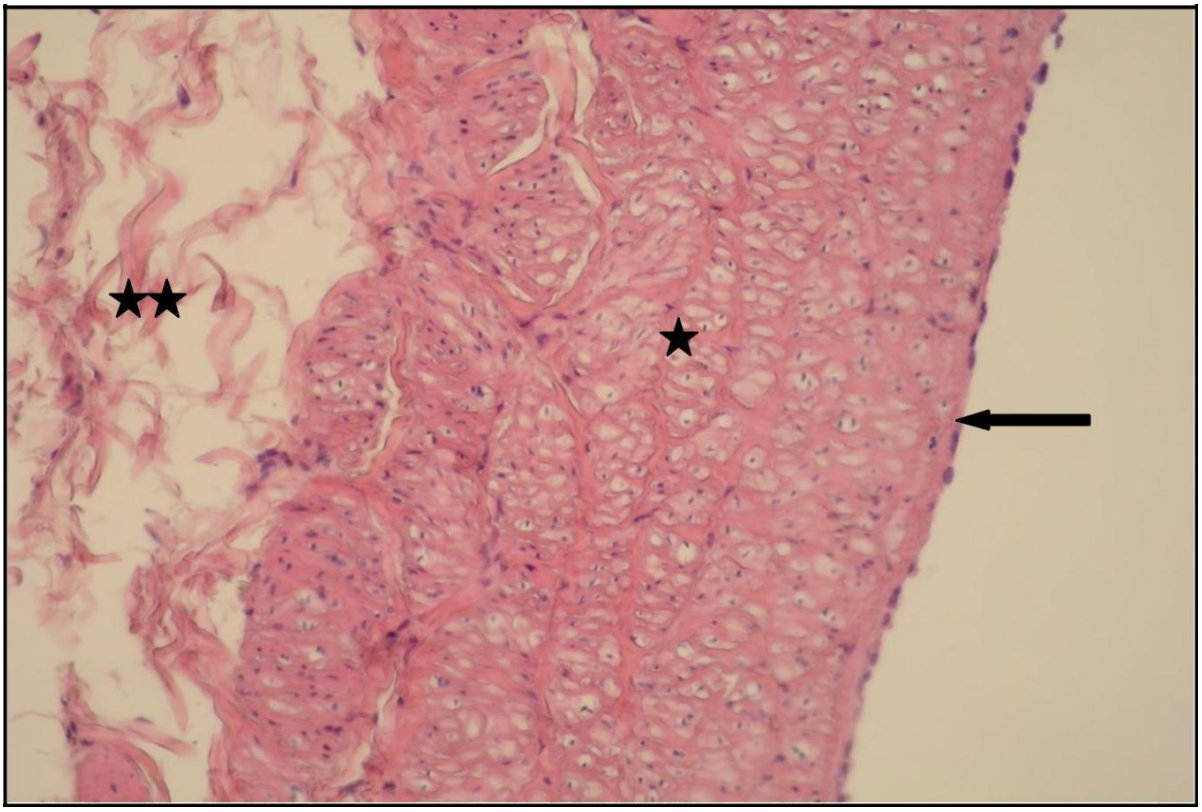

Figure 8. The punctured vein. The arrow points to the intima (endothelium); one star denotes media; two stars denote adventitia. HE, x200.

Mallory and Gomori's histochemical staining demonstrated a loss of their elongated morphology in punctured veins and slightly increased collagen matrix with degradation of elastic fibers on von Gieson's elastic staining (Figure 9), as compared with non-punctured veins (Figure 10).

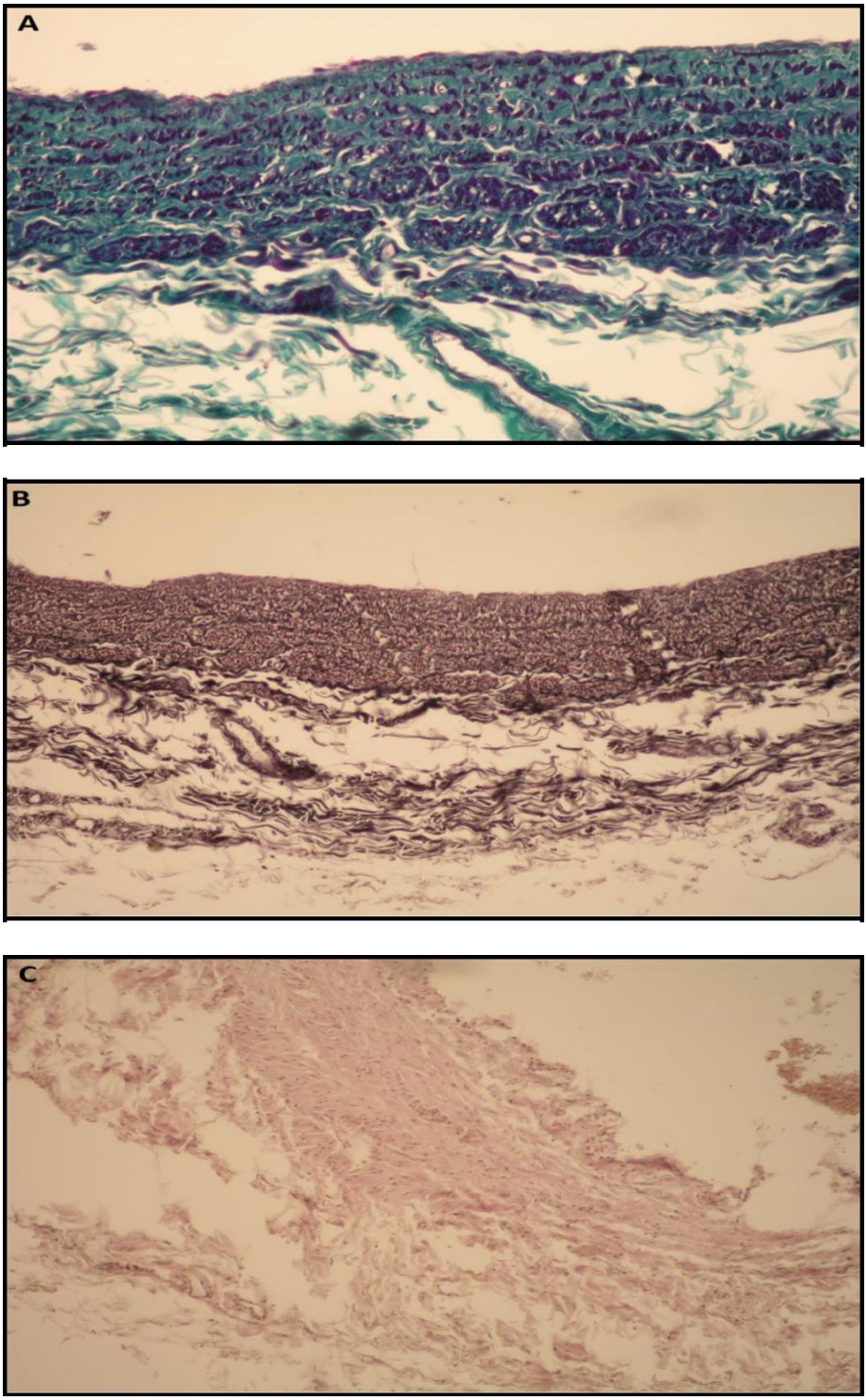


Figure 9. The punctured vein. A. Mallory trichrome staining; B. Gomori's staining; C. von Gieson's staining

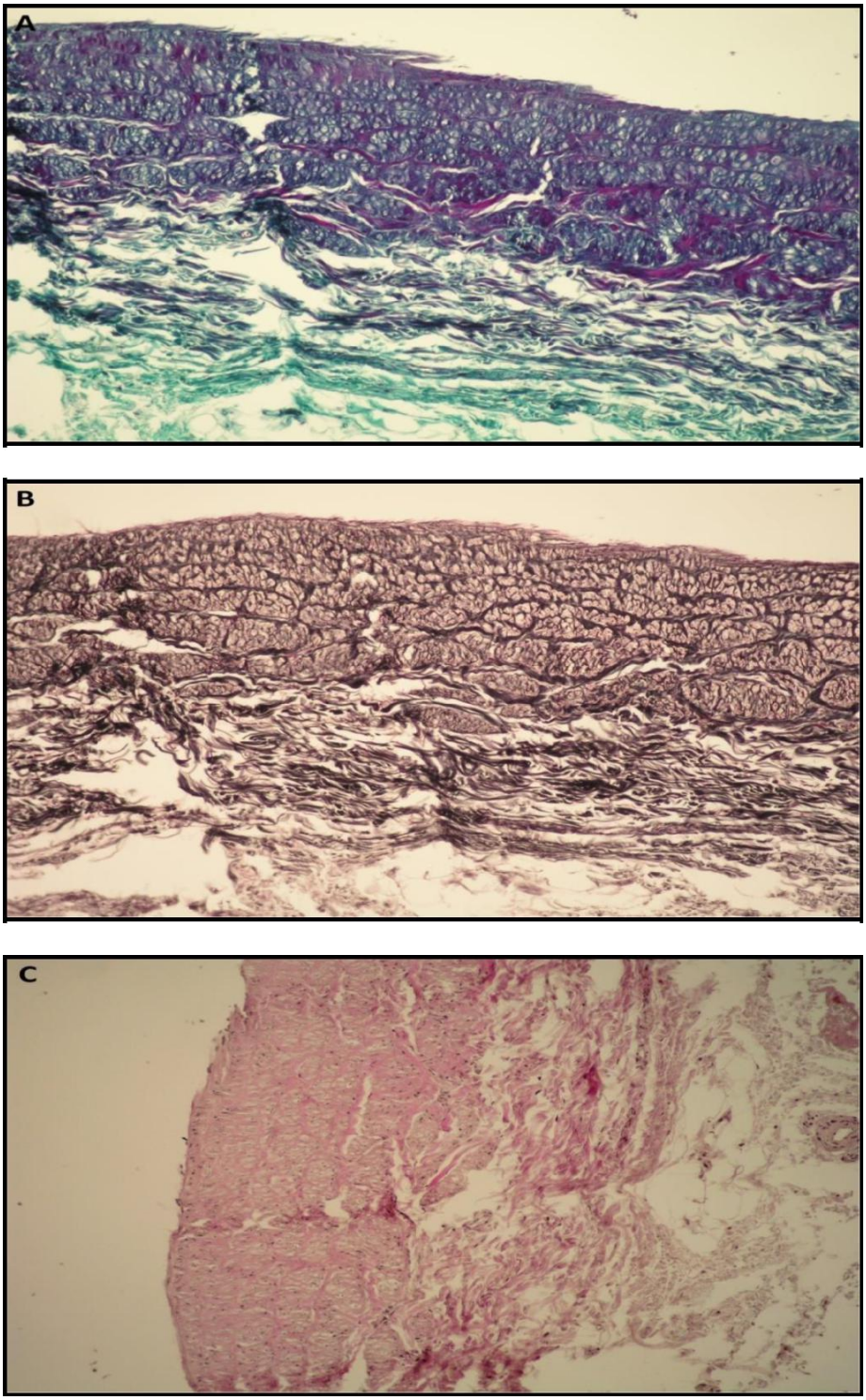


Figure 10. The control vein. A. Mallory trichrome staining; B. Gomori's staining; C. von Gieson's staining

### 5.3. Expression of apoptotic and antiapoptotic markers (IHC)

#### 5.3.1. Immunohistochemical results in the study group

p53 showed no expression or a minimal expression in veins. Bcl-2 showed a minimal and moderate expression. Caspase 3 showed minimal, moderate and maximal expression and Bax showed no expression or minimal and moderate expression (Table 3, Figures 11, 12, 13, 14).

Table 3. Semiquantitative analyses of markers expression in study group

| | **p53** | **Bcl-2** | **caspase 3** | **Bax** |
|---|---|---|---|---|
| - **0%** | 18 | 0 | 0 | 11 |
| - **1-3%*** | 12 | 20 | 6 | 14 |
| - **>3-50%*** | 0 | 10 | 18 | 5 |
| - **>50%*** | 0 | 0 | 6 | 0 |
| **Total** | 30 | 30 | 30 | 30 |

*-minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

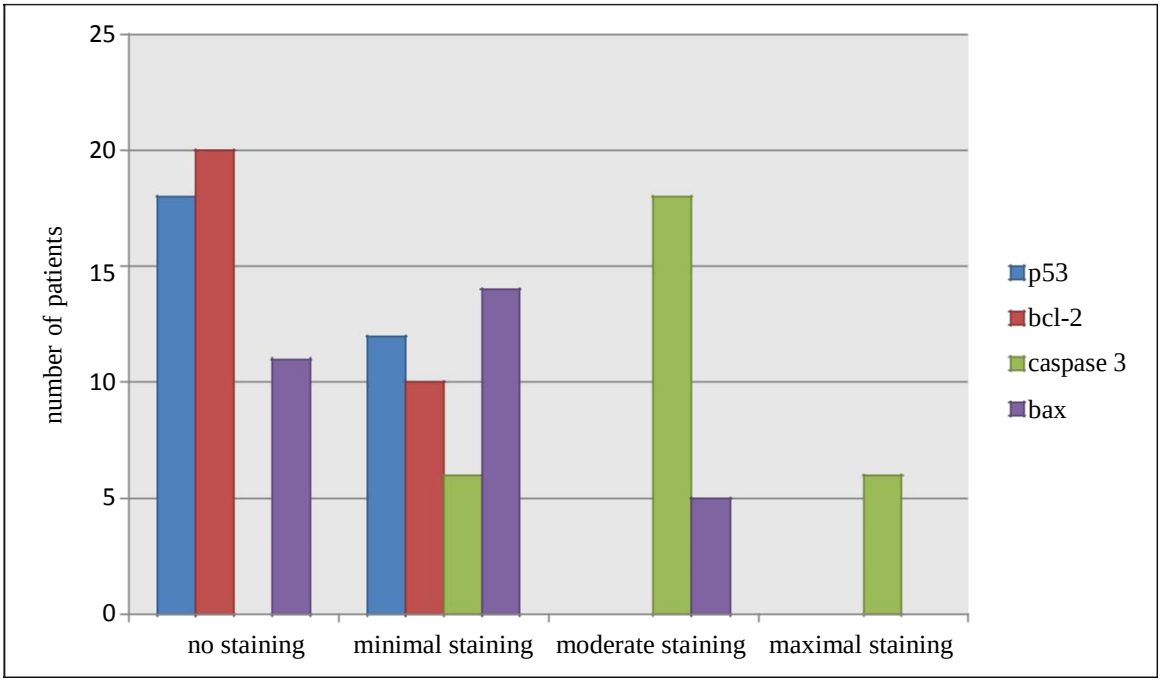


Figure 11. Expression of apoptotic and antiapoptotic markers for the study group
Note: minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

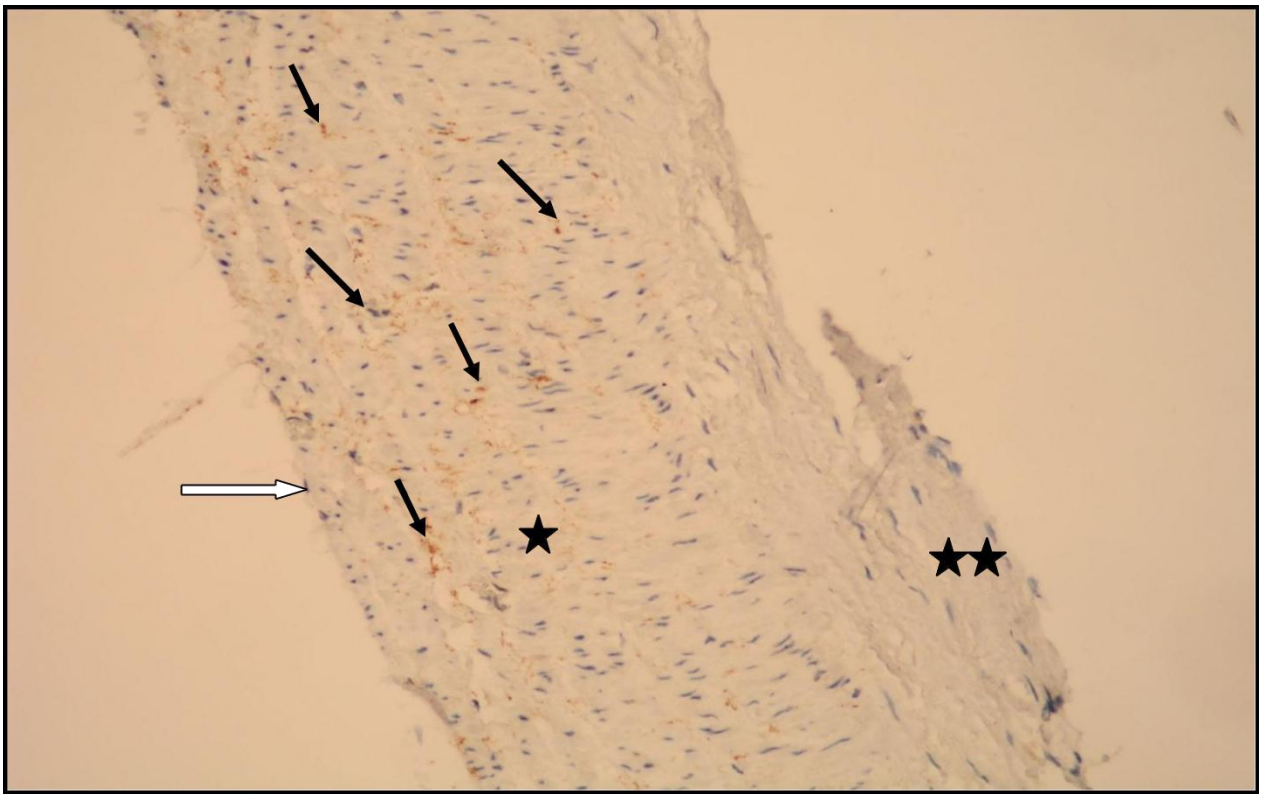

Figure 12. Positive cytoplasmic immunohistochemical staining of Bax in the media and intima of the punctured vein. IHC, DAB, counterstained by hematoxylin, x200. White arrow points to the intima; one star denotes media; two stars denote adventitia; small black arrows point to the positive cytoplasmic staining

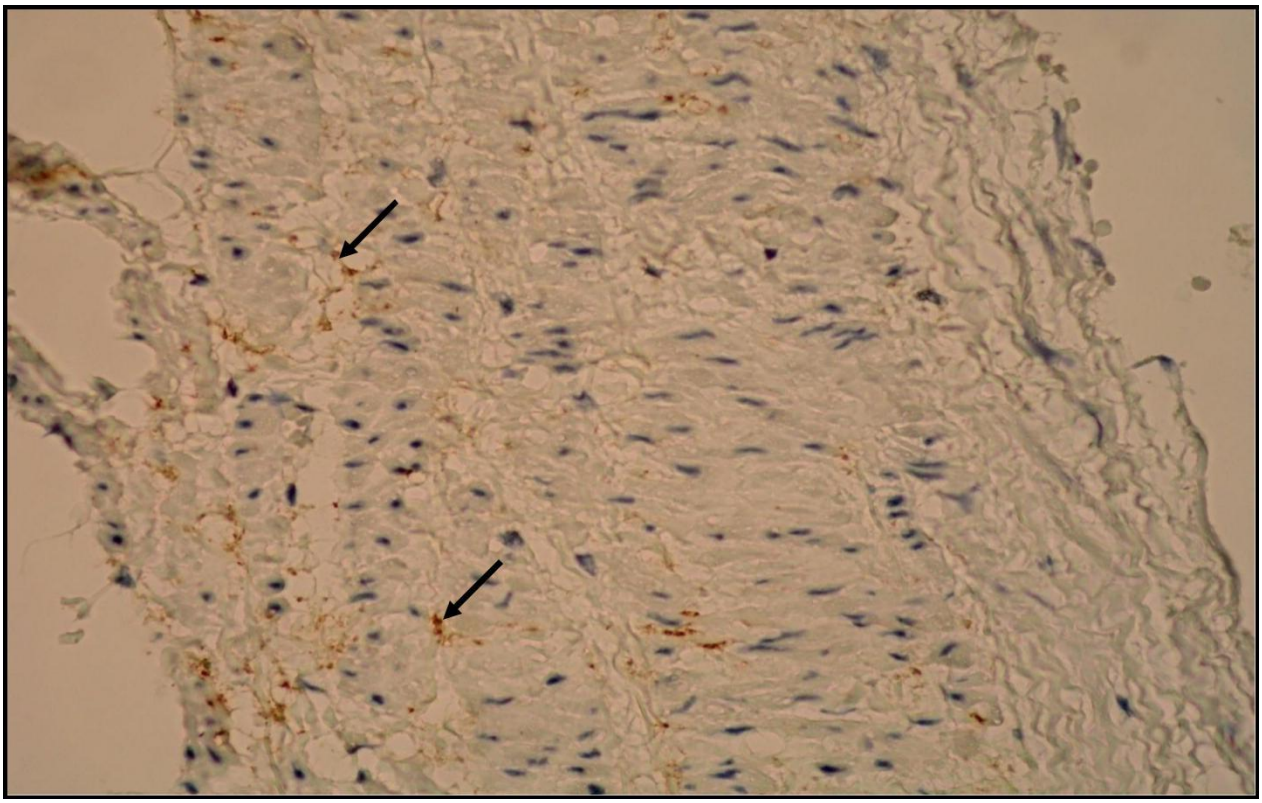

Figure 13. Positive cytoplasmic immunohistochemical staining of caspase 3 in the media and intima of the punctured vein. IHC, DAB counterstained by hematoxylin, x400. The arrow points to the positive cytoplasmic staining.

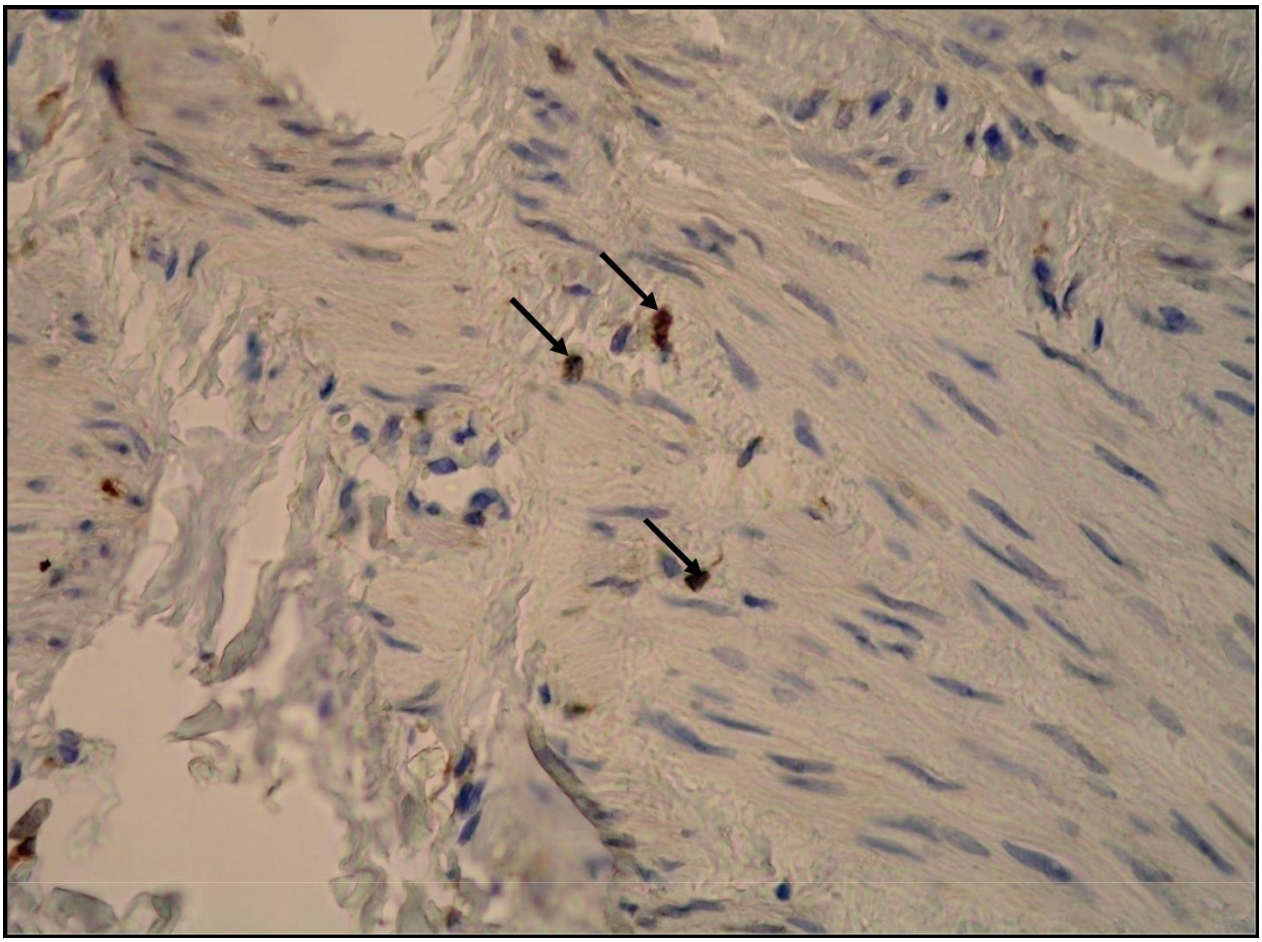

Figure 14. Positive nuclear immunohistochemical staining of p53 in the media of punctured vein,IHC, DAB, counterstained by hematoxylin, x400. The arrow points to the positive nuclear staining.

### 5.3.2. Immunohistochemical results in the control group

p53 showed no or a minimal expression in veins. Bcl-2 showed a minimal, moderate and maximal expression. Caspase 3 showed no or minimal expression and Bax showed no or minimal expression (Table 4, Figures 15,16,17,18).

Table 4. Semiquantitative analyses of markers expression in control group

| | **p53** | **Bcl-2** | **caspase 3** | **Bax** |
|---|---|---|---|---|
| - **0%** | 19 | 0 | 19 | 23 |
| - **1-3%*** | 11 | 3 | 11 | 7 |
| - **>3-50%*** | 0 | 20 | 0 | 0 |
| - **>50%*** | 0 | 7 | 0 | 0 |
| **Total** | 30 | 30 | 30 | 30 |

*-minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

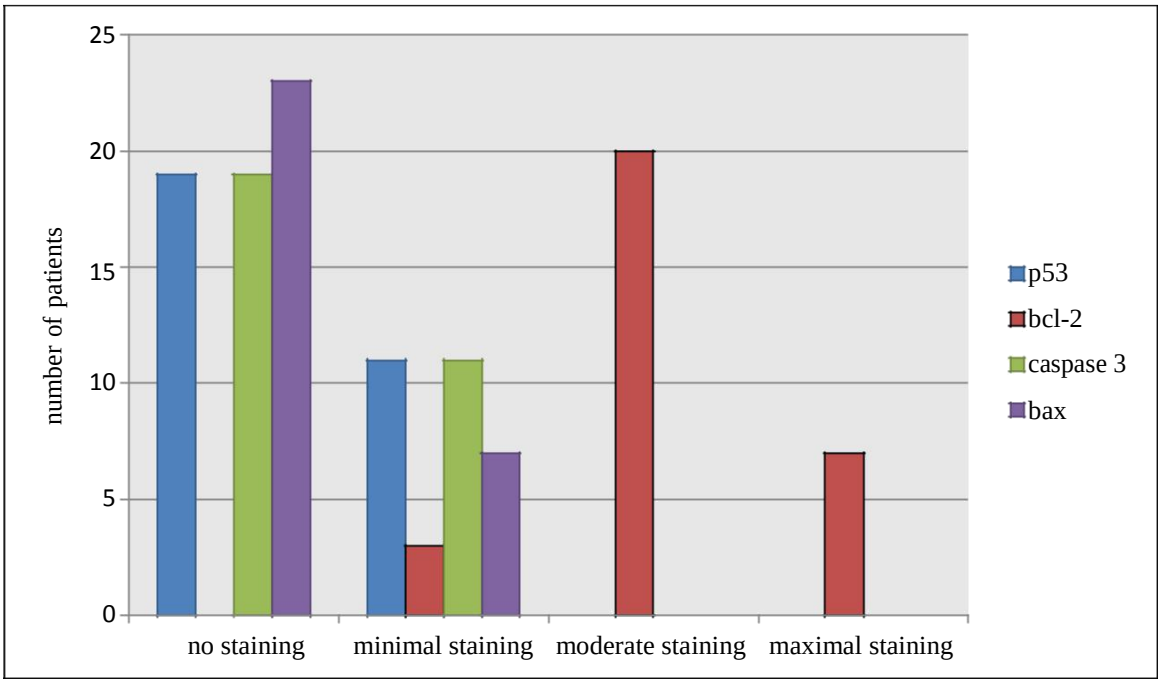


Figure 15. Expression of apoptotic and antiapoptotic markers for the control group
Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells).

Figure 16. Nuclear immunohistochemical positivity of Bcl-2 in the media of the control vein. IHC, DAB, counterstained by hematoxylin, x400. The arrow points to the positive reaction, visible as a dark staining in nucleus (black arrow)

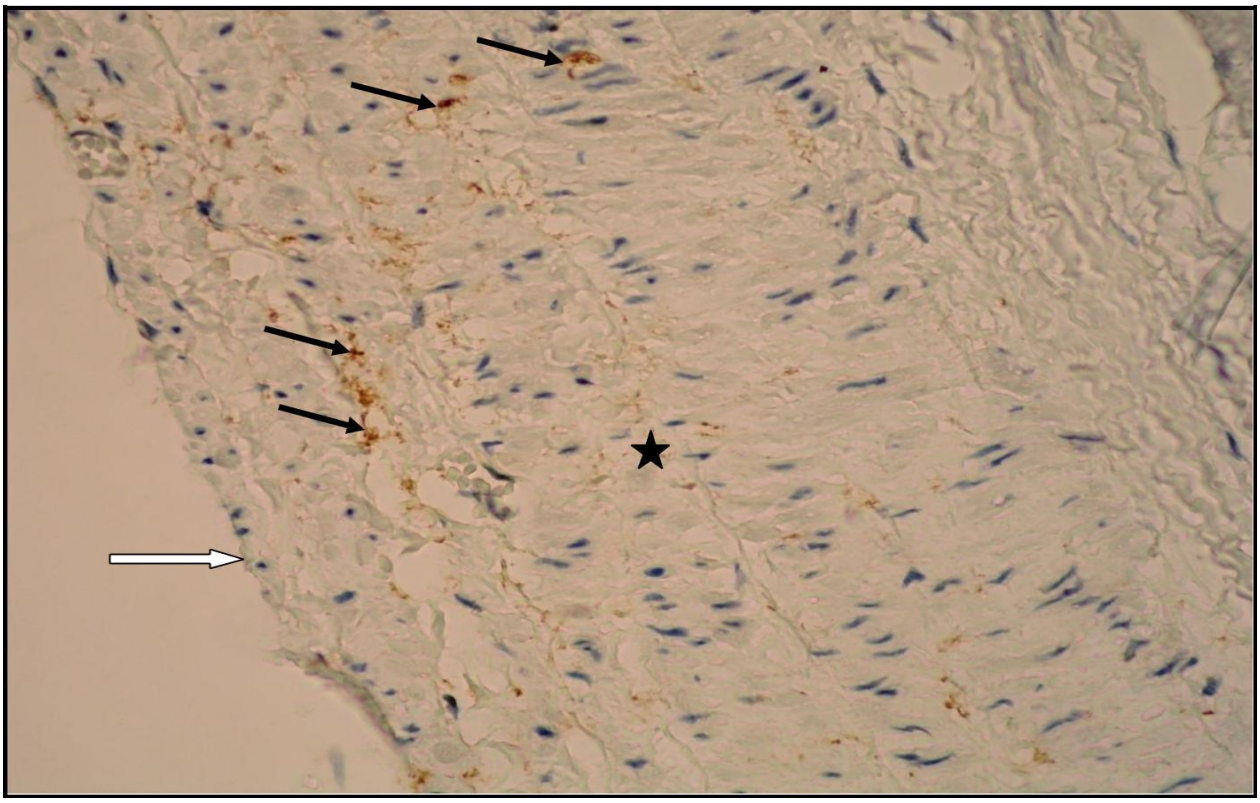

Figure 17. Positive cytoplasmic immunohistochemical staining of caspase 3 in media and intima of the control vein. IHC, DAB, counterstained by hematoxylin, x400. White arrow shows intima; black arrows show positive cytoplasmic staining; star shows media.

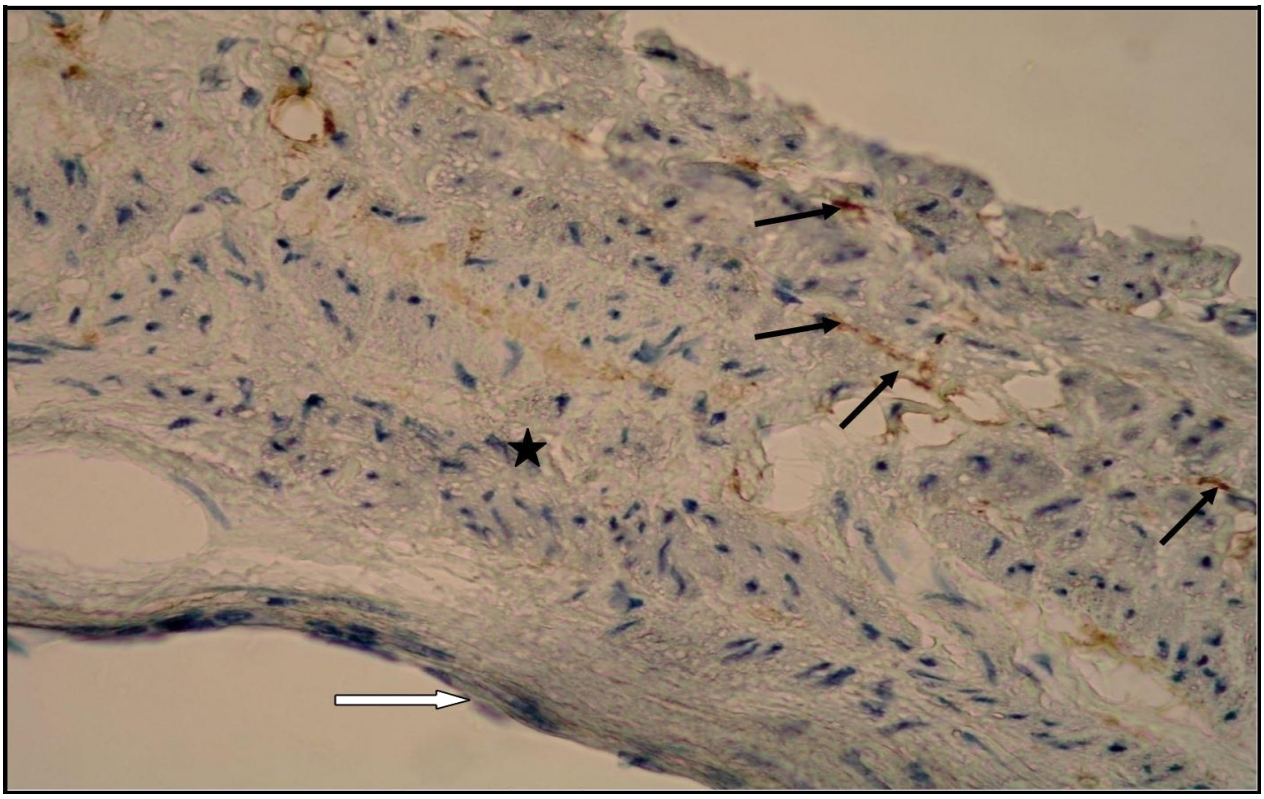

Figure 18. Positive cytoplasmic immunohistochemical staining of caspase 3 in media and intima of the control vein. IHC, DAB, counterstained with hematoxylin, x400. White arrow points to the intima; black arrows show positive cytoplasmic staining; star denotes media.

### 5.3.3. Comparison of immunohistochemical results between groups

There were significant differences between the two groups for the expression of Bcl-2, caspase 3 and Bax. Caspase 3 and Bax expression in the study group was increased compared with the control group ($p<0.01$ for all). Bcl-2 expression in the study group was decreased compared with the control group ($p<0.01$). p53 showed no significant differences between the two groups ($p=0.791$) (Table 5, Figures 19,20,21,22).

Table 5. Comparison of the apoptotic and antiapoptotic markers expression between the study group and control group

| | | Study group | Control group | p Value |
|---|---|---|---|---|
| **p53** | | | | 0.791* |
| | no staining | 18 | 19 | |
| | minimal staining | 12 | 11 | |
| | moderate staining | 0 | 0 | |
| | maximal staining | 0 | 0 | |
| **Bcl-2** | | | | <0.001* |
| | no staining | 0 | 0 | |
| | minimal staining | 20 | 3 | |
| | moderate staining | 10 | 20 | |
| | maximal staining | 0 | 7 | |
| **caspase 3** | | | | <0.001* |
| | no staining | 0 | 19 | |
| | minimal staining | 6 | 11 | |
| | moderate staining | 18 | 0 | |
| | maximal staining | 6 | 0 | |
| **Bax** | | | | <0.002* |
| | no staining | 11 | 23 | |
| | minimal staining | 14 | 7 | |
| | moderate staining | 5 | 0 | |
| | maximal staining | 0 | 0 | |

*Fischer's Exact Test

Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

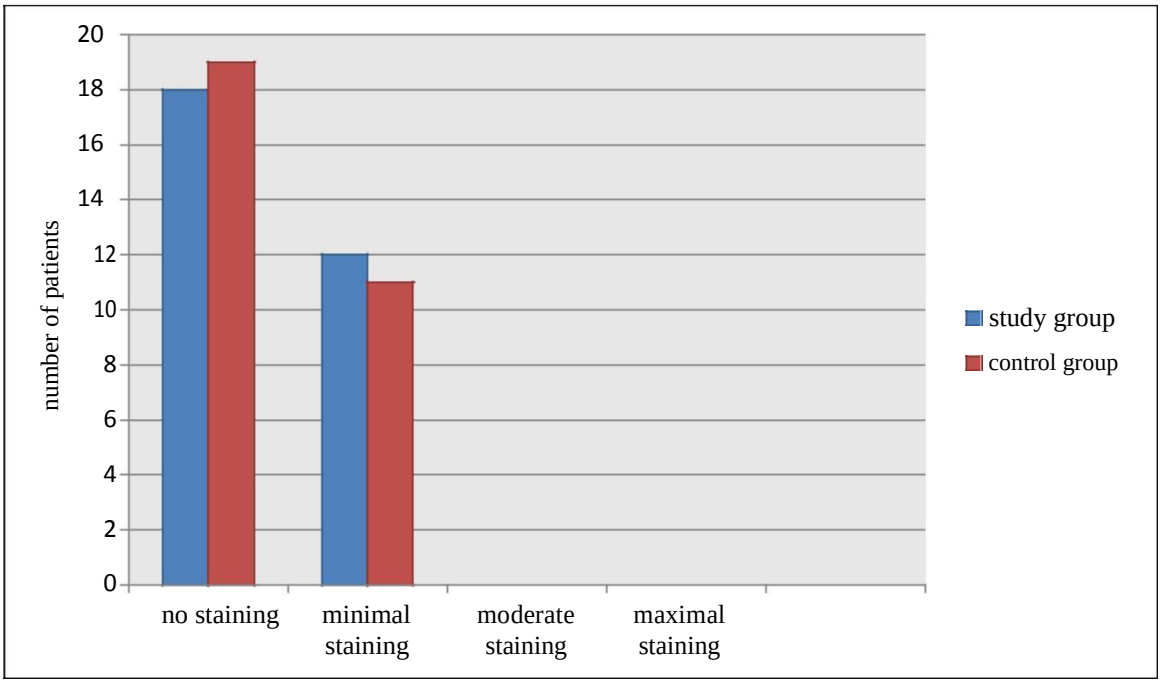


Figure 19. Expression of p53 (IHC staining)
Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

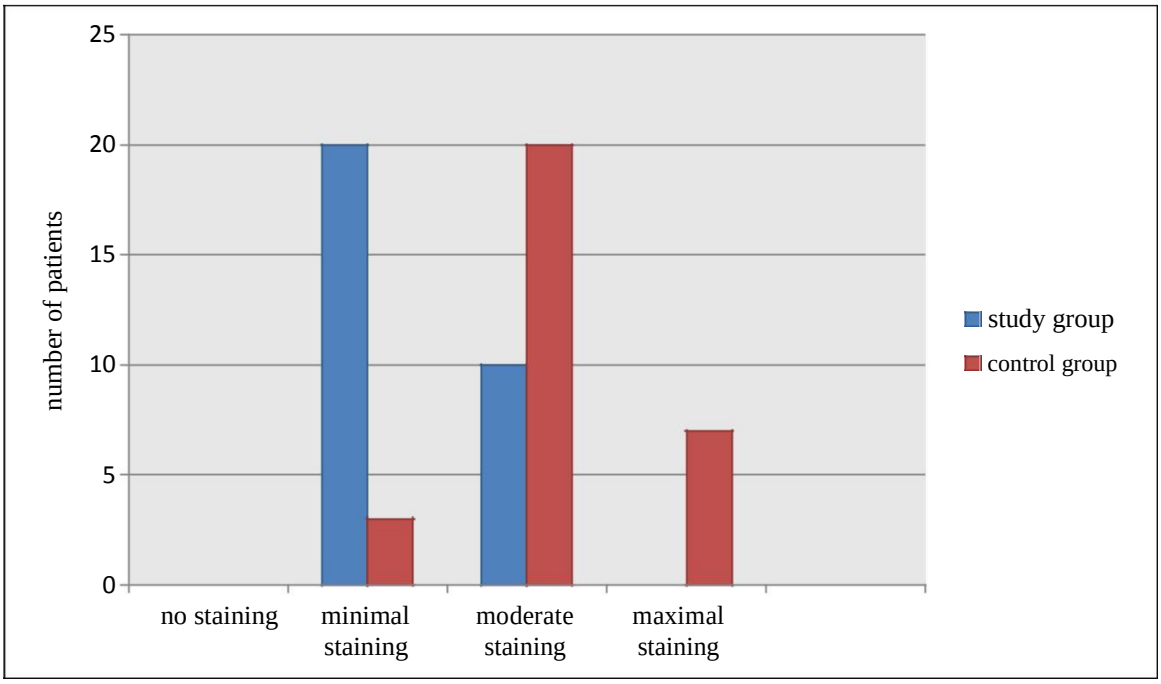


Figure 20. Expression of Bcl-2 (IHC staining)
Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

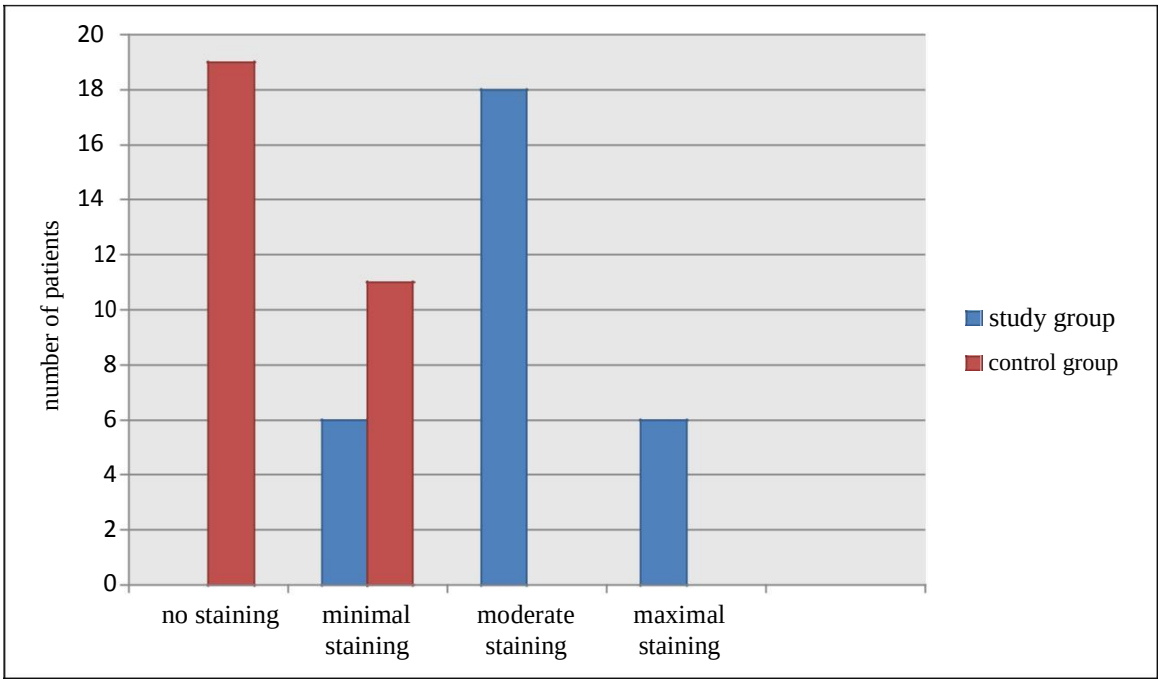


Figure 21. Expression of caspase 3 (IHC staining)
Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

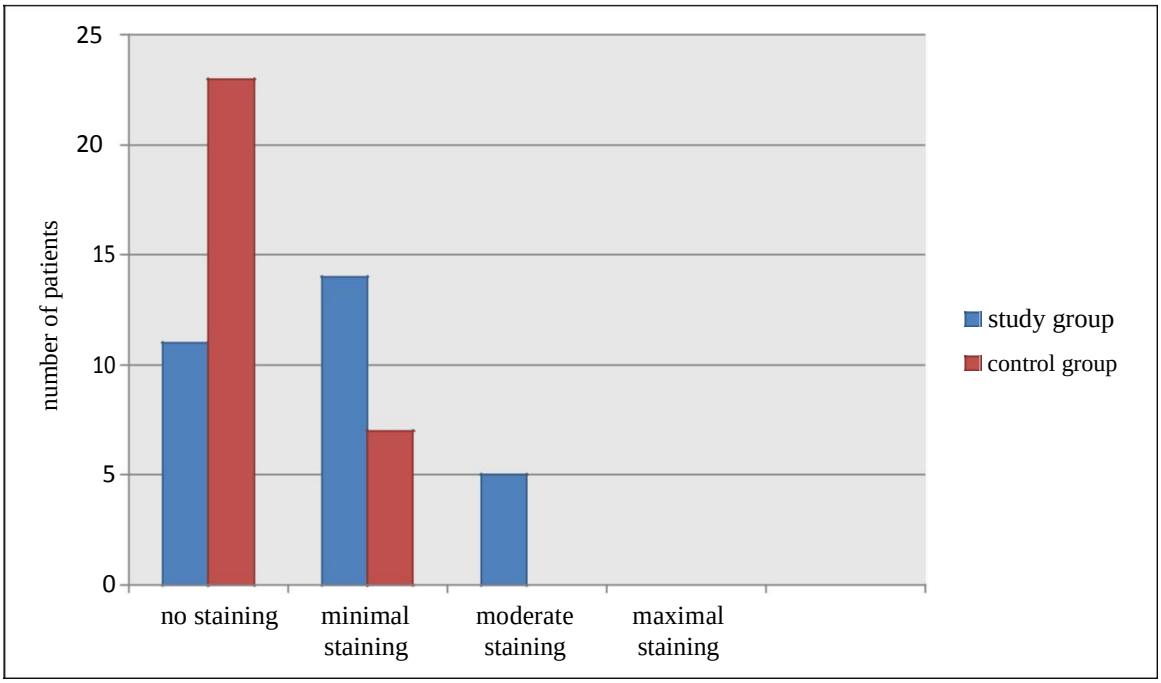


Figure 22. Expression of Bax (IHC staining)
Note minimal staining (1-3% positive cells), moderate staining (>3-50% positive cells), maximal staining (>50-100% positive cells)

### 5.4. Clinical characteristics of patients after the one-year follow-up

During the one-year follow-up, fistula failure occurred in eight patients of the study group, and in two patients of the control group. Five patients with a functional fistula in the study group and three patients in the control group were lost during follow-up (Table 6).

Table 6. Clinical characteristics during one-year follow-up

| | Group I | Group II | Total |
|---|---|---|---|
| Death | 5 | 3 | 8 |
| Fistula failure | 8 | 2 | 10 |

There were statistically significant differences in fistula failure between the study group and control group (26.7% *versus* 6.7%, p=0.038) (Table 7, Figure 23).

Table 7. Fistula failure within study group (Group I) and control group (Group II)

| | | | Group I study group | Group II control group | Total |
|---|---|---|---|---|---|
| **FISTULA FAILURE** | **NO** | Number | 22 | 28 | 50 |
| | | % within fistula failure | 44.0% | 56.00% | 100% |
| | | % within group | 73.3% | 93.3% | 83.3% |
| | | % of total | 36.7% | 46.7% | 83.3% |
| | **YES** | Number | 8 | 2 | 10 |
| | | % within fistula failure | 80.0% | 20.0% | 100% |
| | | % within group | 26.7% | 6.7% | 16.7% |
| | | % of total | 13.3% | 3.3% | 16.7% |
| Total | | Number | 30 | 30 | 60 |
| | | % within fistula failure | 50.0% | 50.0% | 100% |
| | | % within group | 100% | 100% | 100% |
| | | % of total | 50.0% | 50.0% | 100% |

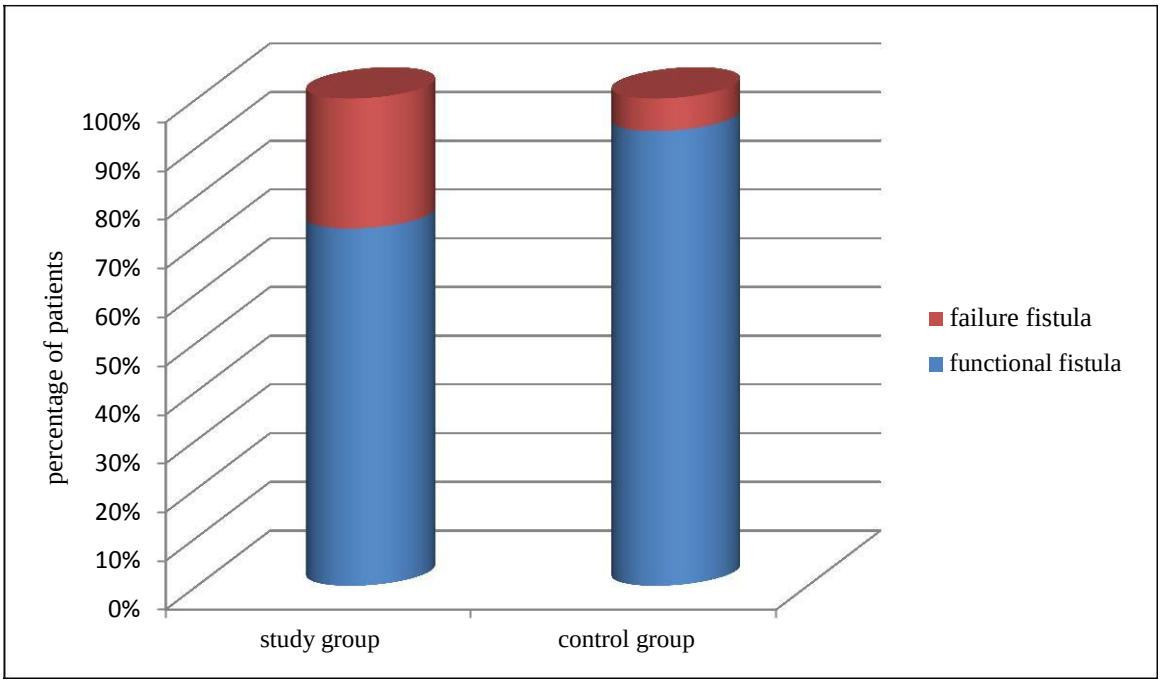


Figure 23. Comparison of fistula failure between the study group and control group

There was also a direct correlation between p53 and central vein catheter, which means patients who were performing dialysis by CVC, before fistula placement. Statistically significant differences were found in correlation with two groups together, p=0.011, and in correlation with the control group, p=0.015. But, there was no statistically significant difference in correlation with the study group, p=0.266.

# 6. DISCUSSION

## 6.1. Clinical procedures and patients characteristics

Nowadays, the number of patients with end stage kidney disease is continuously growing. When performing the vascular access, the surgeon must know that nothing less than perfection is acceptable (52,53,92,96).

Considering the fact that no research has been done yet on the veins of the upper extremities, the only way to achieve results was to study the veins which are used for hemodialysis access. Our interest was to investigate the apoptosis of these veins.

To our knowledge, no published studies have before evaluated apoptosis of veins in upper extremities, or in patients who will undergo a surgery for arteriovenous fistulas for hemodialysis access (13,31,39,124).

Cell death is important for both development and tissue homeostasis in the adult. Abnormalities in the control of cell death contribute to a variety of diseases such as atherosclerosis, aneurysm formation, ischemic cardiomyopathies, infarction, varicose veins, tumors, etc. (125,126,127). Numerous studies have reported on apoptosis in atherosclerotic plaque, varicose vein, occluded saphenous vein graft, myocytes, etc. (14,38,124,128).

Increased levels of VSMC apoptosis are seen in mature atherosclerotic plaques compared with control vessels, and in unstable angina compared with stable angina (39,124). Previously, several studies have reported on apoptosis of varicose veins of the lower extremities (25,26,30,31,46,48,125,126,129,130).

As our Clinic is a reference center in the region, we place all arteriovenous accesses for hemodialysis. We ourselves perform the arterial and venous Doppler US.

There are still some difficulties in managing vascular access, creating it and dealing with all of its complications. With patients with serious access problems, there is a need to determine the patency of arteries and central veins by arteriography or venography. Reducing factors that aggravate stenosis will lead to a reduced rate of fistula failure (77,80,81,94,96,116).

Our research was conducted on patients who for the first time had arteriovenous fistula placed for hemodialysis access. The same patients were followed over a year, to assess the function of that fistula.

In the present study, the median age of group 1 was 63.50 years, and the male/female distribution was 18/12. This did not differ from group II, which involved patients with median age 63.00 years, and with a male to female ratio of 20/10. The study of Simovart et al. revealed that in varicose veins ECs and SMCs apoptosis increased with advancing age. Urbanek et al. described an increase of the apoptotic index in the media of varicose veins of patients younger than 50 years, but not in the older age patients (30,46,51,124).

When dealing with the issue of vascular access in diabetic patients, late referral has a special impact on diabetes, since it is common opinion that dialysis should be started earlier in these patients. We could not detect any significant difference between the groups, as to diabetes as a comorbidity. All our patients were on insulin therapy, which is in agreement with the fact of late referral. Therefore, practitioners must be alerted as to their key role in referring diabetic patients with nephropathy to the nephrologist on time. Venous sparing, starting from the very beginning of renal failure, is another rule, to be strictly adhered to by health professionals: very often forearm veins are repeatedly cannulated for infusions and blood drawings in candidates for hemodialysis (69,73,76,100,105,123). Sedlacek et al. observed similar maturation rates of fistulas among diabetic and non-diabetic patients (55,94).

Also in terms of other associated diseases, such as hypertension, hematologic disease, cerebrovascular, pulmonary, rheumatologic disease and cancer, we did not observe statistical difference as compared between the two groups.

Chronic hemodialysis patients are at high risk of infection because the process of hemodialysis requires vascular access for prolonged periods. In an environment where multiple patients receive dialysis, repeated opportunities exist for transmission of infection, either through person-to-person, directly or indirectly via contaminated devices, equipment, environmental surfaces, or hands of personnel (84,105,109,131). In our study, we observed a very small number of patients with positive HBsAg (two patients in group I; two patients in group 2), and positive HCV (one patient in group 1; two patients in group II). Meanwhile no patient was HIV positive. It also confirms the fact that patients in our research just started or were still not on dialysis, because the study included only those patients who for the first time had arteriovenous fistula placed. In our study, there were 19 patients in group I, and 20 patients in group II, who at the time of entering the study were undergoing hemodialysis through the central vein catheter. We did not find statistically significant differences between the groups in the number of placed catheters and the vein in which the catheter was placed. Smaller number of cases had catheter placed in the subclavian vein (two patients out of 19 with CVC, in group I; three patients out of 19 with CVC, in group II). This is consistent with the data from the literature, to place CVC in the subclavian vein as little as possible, in order that a possible subclavian vein thrombosis could compromise fistula placed in that area. Duplex ultrasonography assessment of veins is frequently used before a hemodialysis arteriovenous fistula placement. Accurate determination of venous diameter is important, because this parameter has been demonstrated to correlate with long term AVF patency. Smith et al. showed that the present guidelines suggest a minimum diameter of 2 mm

for a radial artery, and a minimum diameter of 2 mm for a cephalic vein for successful AVF placement at the wrist. These measures have been evidenced by meta-analysis demonstrating significant differences in fistula success rates between diameters >2.0 and <2.0 mm (70,86,94,101,122,132). A previous study found that the only parameter that affects maturation time of brachiobasilic fistula is the vein diameter. AV fistulas using basilic veins with diameters larger than 3mm could be used for hemodialysis in the short term (90,103,118).

Van der Veer et al. showed that less than half of the patients were estimated to receive routine diagnostic imaging of the vessel prior to access creation. As potential reasons they suggested skepticism among vascular surgeons regarding the benefits of imaging, limited access to equipment and expertise, and lack of reimbursement. On the other hand, some other reports observed that while using preoperative physical examination alone, only a small proportion of patients (0 to 34%) received a fistula, rather than a graft. Following the introduction of routine preoperative vascular mapping, the proportion of patients receiving fistulas increased to 63 to 100% (55,78,133). Vessel diameters in our study were generally well above the widely recommended minimum artery and vein diameters of 2.0 and 2.5 mm, respectively. Neither group had surgical complications or thrombosis in the immediate postoperative period. In the present study, we estimated the condition of artery and vein, pre- and intraoperative, and we did not find a significant difference in results obtained from the comparison between the two groups.

**6.2. Apoptosis in blood vessels**

Previous studies of apoptosis in blood vessels often were opposed to each other. Isner et al. performed immunohistochemical staining studies on specimens retrieved from patients undergoing directional atherectomy for primary atherosclerotic lesions or recurrent arterial narrowing after percutaneous revascularization or restenosis. They documented that specimens

retrieved from patients with restenosis were more frequently observed to contain foci of apoptosis than were specimens retrieved from patients with primary atherosclerotic lesions (40,43). Ducasse et al, studied a group of patients who had previously undergone varicose vein surgery, which preserved the saphenous trunk. Findings in that group were identical to those in patients with primary varices. They suggested that the abnormal apoptosis is irreversible once it affects the venous system (26,125). Furthermore, a previous study concluded that the apoptosis of ECs and SMCs in the walls of varicose veins maybe differently regulated or these cells react differently to apoptotic stimuli (51). Apoptosis has been verified in various cardiovascular diseases, such as arrhythmogenic right ventricular dysplasia, acute myocardial infarction, and congestive heart failure (14,124,128,134).

Recent studies on apoptosis in the cardiovascular system stem from the hope that understanding the mechanism of apoptosis in cardiac myocytes may provide new strategies to prevent myocyte loss at major cardiac disease states such as ischemic heart disease and congestive heart failure (19,135). Apoptosis due to immune mechanism could be a major cause of myocarditis. During cardiac surgery with cardiopulmonary bypass, mechanical stress and hypothermia induce myocardial apoptosis, too (14). Deblois et al. proposed that cardiovascular hyperplasia may be reversed via therapeutic apoptosis induction with drugs that are safe and already used in the clinic (124,134). Also, Kovačević et al. concluded that if the apoptotic cell death significantly contributes to the expansion and rupture of abdominal aortic aneurysm, the hypothesis is that aggressive medical antiapoptotic treatment with high doses of appropriate drugs could decrease the apoptotic index of smooth muscle cells, reduce the aneurysm expansion and prevent rupture (38). Furthermore, occlusion of saphenous vein grafts is a major problem after coronary artery bypass grafting. Previous studies showed that vein graft disease, an accelerated form of

atherosclerosis, shares etiologic mechanisms with restenosis after angioplasty. Their data could open new therapeutic strategies with the goal of improving SMC survival, and hence increasing plaque stability and reducing vein graft occlusion rates (42,136). Most histopathological studies describe intimal hyperplasia and accelerated atherosclerosis in the vein grafts as the reason for occlusion (136). Recent studies suggest that in the cardiovascular setting, apoptosis is one of the many processes controlled by statins, because they are thought to stabilize plaques and help prevent the development of acute coronary syndromes triggered by plaque disruption. Plaque breakdown and rupture seem to be related to SMC apoptosis (38,42,124).

There is no totally specific apoptotic marker that solely detects apoptotic cells, so that a combination of techniques should always be used to detect apoptosis. The TUNEL staining method is now the most widely used marker for apoptosis in cardiomyocytes. A weakness of this method is that DNA fragmentation is not 100% specific for the apoptotic type of cell death (5,20,42). In many published myocardial sections with positive TUNEL staining, apoptotic cells and nuclei exhibit a strikingly normal morphology and only few reports provide evidence for ultrastructural alterations such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies and phagocytosis of cell remnants (135). All these facts increased doubts that evidence for apoptosis solely based on TUNEL staining and even DNA laddering may not suffice to prove apoptotic cell death. DNA damage is not a unique feature of apoptosis, but can occur in necrosis, during repair of reversibly damaged DNA and postmortem autolysis (23,42). Previous studies for immunohistochemical analysis of apoptosis in varicose veins and thrombosed venous grafts, except TUNEL method, used to localize the expression of Bax, caspase 3, caspase 8, caspase 9, Bcl-2, Bcl-xs, Bcl-6, Ki-67, p53, DNA laddering, Annexin V, Elisa, Western blot (5,26,30,42,48,129,130).

In this study, we selected measurement of Bax as a promoter of apoptosis, Bcl-2 as an antiapoptotic marker, caspase 3 as a part of execution phase of apoptosis, and p53 to evaluate its role in apoptosis of the veins of the upper extremities.

The process was seen in the different layers of the vein wall with the most prominent manifestation in the media of the cephalic vein used for AVF.

Bax expresses proapoptotic activity, and in this study it was significantly increased, in the group of previously punctured veins. Our results are compatible in part with those of Urbanek at al., which showed increased Bax in the distal part of the varicose vein, but are opposite to the findings of Ascher et al., that immunoreactivity for Bax is significantly higher in the normal veins (30,129,137). They showed total absence of immmunopositivity for Bax in the adventitia of all varicose vein specimens. This is in contrast to findings in the media and intima. The entry of SMCs into the apoptotic pathway is regulated by Bax, which is downregulated in varicose veins in comparison with healthy veins (125,129). In the study of Ducasse et al., varicose veins contained fewer Bax positive cells than healthy veins (26,125). By contrast, in the study of Filis et al, one of the main results was increase of Bax expression in varicose veins compared with normal veins (48). Also, Simovart et al. showed lower levels of Bax in the intima and media of varicose veins of patients over 50 years of age, which could be explained by shifts in the sensitivity of cells to apoptosis caused by aging, or by changed signaling during the advanced stage of the pathology (51). Hayakawa et al. have reported that expression of Bax protein was increased according to the progression of atherosclerosis, whereas expression of Bcl-2 was not observed. Discrepancy exists in the literature concerning the expression of Bcl-2 in human medial SMC (2).

Our results are compatible with the findings by Isner et al, regarding immunohistochemical evidence of the Bcl-2 protein. Bcl-2 was identified in sections of nonpunctured veins, with significant difference ($p<0.001$), when compared with previously punctured veins. This finding is similar to the study of Isner et al. in control sections of normal vascular tissues excised intraoperatively, including internal mammary artery and saphenous vein (43). Some investigators reported a lack of immunopositivity for Bcl-2 in atherosclerotic lesions of primary and restenotic types (43,137).

Filis et al., as well as Ascher et al., could not detect Bcl-2 expression in varicose veins and concluded that this is maybe due to the lack of specificity of the antibody used to cell types in the vein tissue. Ascher et al. showed that programmed cell death is inhibited in varicose veins (48,129,137). Ducasse et al. found no significant difference for Bcl-2 proteins in patients with varicose veins and controls (26,125).

The tumor suppressor p53 has been shown to induce apoptosis when overexpressed in some cultured cells (42,48). Traditionally, p53 has been described as a nuclear protein, some recent studies indicate a new role for p53 in the cytoplasm and specifically at the mitochondria. Hammond et al. found that p53 overexpression is as a response to hypoxia. Based on their data, Filis et al. did p53 examination in their study for apoptosis in varicose veins, in order to investigate a possible link between low oxygen tension, which was observed in varicose vein disease, and p53 overexpression as a response to hypoxia (48,138). We do not have data for hypoxia at veins after venipuncture. This problem remains to be further explored in the future.

In the present study, we could not detect any p53 immunopositivity difference between groups I and II. However, a previous study observed that immunopositivity for p53 was in fact recognized in a subset of restenotic lesions including those with apoptosis. It was identified in atherectomy

specimens retrieved from restenotic but not primary lesions (43,124). In the study of Bartels et al., the proto-oncogene p53 was exclusively detected in occluded vein grafts (42). Filis et al. in their study on apoptosis in varicose veins found expression of p53 only in the tributary and the distal GSV of control group that consisted of patients with healthy GSV, used for bypass grafting in open heart surgery. Also, Urbanek at al. found increased p53 expression in the distal GSV in young patients. They suggested that elderly patients show increased structural vein wall changes, and hence, p53 expression is not evident (30,48). Jacob et al. in their study indicate increased expression of p53 in the nuclei of vascular smooth muscle cells in the media of injured rat carotid arteries, compared with the controls (139).

Earlier studies have demonstrated that activation and cleavage of specific cellular polypeptides, termed caspases, play critical roles in the initiation and execution of apoptosis (13,23,35,46,48,124). In this study, we observed enhanced caspase 3 expression in group I compared with group II, with statistically significant difference ($p<0.001$). Filis et al. observed a significant difference in caspase 3 immunopositivity between varicose vein group and control group. It suggests an active apoptotic state in varicose veins (48).

**6.3. Fistula failure**

Smith et al. showed that AVF placement before dialysis is initiated may allow maturation in a biochemical environment preferable to that present once dialysis has commenced. Uremia and dialysis itself are associated with increased level of oxidative stress, precipitating endothelial dysfunction, neointimal hyperplasia and AVF stenosis. Samples of brachial vein obtained during access placement in 15 uremic patients showed significantly greater intimal and medial widths in patients who had received >6 months of dialysis compared with those who had received <6 months of treatment (86).

The inflammatory environment in HD patients has been attributed to the incomplete removal of uremic toxins that promote inflammatory reactions but also to the HD procedure itself. Atamaniuk et al. found that cfDNA released from blood cells by ongoing apoptosis is abundantly present in the plasma of HD patients. They showed that the plasma of HD patients mimicked the capacity of cfDNA to induce IL-6 in human monocytes indicating that this process may contribute to the proinflammatory environment observed in HD patients (3,60,65).

Neointimal hyperplasia and impaired dilatation are important contributors to arteriovenous fistula failure. Vein dilation after arterializations is a part of a physiological AVF maturation process, which makes recurrent cannulation for dialysis possible. Neointimal hyperplasia results from proliferation of smooth muscle cells combined with matrix deposition. There is evidence that surgical trauma and modified hemodynamics are associated with endothelial and SMC damage (40,41,91,103,119). That was the reason why we took a sample of vein, very carefully, without previous dilatation. Langer et al. in their study presumed that it was the uremic environment rather than indirect effects that caused the exacerbation of neointimal hyperplasia and calcification within the AVF (45).

In the present study, we observed a significant difference in caspase 3 and Bax immunopositivity between group I and group II. This finding suggests an active apoptotic state in previously punctured veins. And, as a result of this increased apoptotic activity, we think that it comes to the biggest failure of fistulas in patients with previously punctured native veins (26.7% vs 6.7%) with a significant difference ($p=0.038$). However, even with that finding, we cannot conclude that apoptosis is the main cause of fistula failure. Our results support the hypothesis that apoptosis may correlate positively with previous venipuncture and failure of arteriovenous fistula. Limitations of our study are the small number of patients.

The association we have found between previously punctured veins and apoptosis, indicates the role that venipuncture plays in the development of apoptosis, but it does not indicate the role that apoptosis itself plays in the failure of AFV, after their puncture for hemodialysis. Further research is required to investigate this point.

Also, we found a direct correlation between p53 and CVC, meaning patients who were performing dialysis by CVC, before fistula placement. Statistically significant differences were found in correlation with two groups together, p=0.011, and in correlation with the control group, p=0.015. But, there was no statistically significant difference in correlation with the study group, p=0.266. Since our sample was small, this remains to be further explored.

We are in the midst of fundamental changes in the way we address the clinical problem of hemodialysis vascular access dysfunction. The purpose of this last section is both to summarize where we stand and to make suggestions for future scientific advances in this field (119).

In the future, we are interested to continue the study on thrombosed AVF, considering that maybe an acute thrombotic event in the early postoperative phase forms the basis for a total and irreversible vein occlusion. Furthermore, it will be very interesting to research if long time uremia, and hypotension during or after hemodialysis, is one of the reasons for thrombosed AV fistulas. This will also help to define potential targets for future intervention.

## 7. CONCLUSIONS

- We did not observe any significant differences between the study group and control group, in age, sex, and comorbidities, before AVF placement.
- This study provides evidence that previously punctured native veins used for hemodialysis access exhibit increased apoptotic activity, by means of increased caspase 3 and Bax, compared with nonpunctured veins.
- We found a significant decrease of Bcl-2, as an antiapoptotic marker, in the study group, compared with the control group.
- Our results were not supportive of increased p53 expression in the study group compared with the control group.
- This study found a significant difference of p53 between patients who were on dialysis before AVF placement, who were on hemodialysis through central vein catheter, and patients who were not on dialysis before fistula placement, in both groups. A significant difference was found in both groups together and separately in the control group, but not separately in the study group. This is a very useful piece of information, to investigate further research in this point.
- The positive apoptotic findings were not related to patient's age, sex, or comorbidities.
- Patients with increased apoptosis showed increased fistula failure, suggesting an association between apoptosis and fistula failure.
- The association we found between apoptosis and fistula failure, indicates the role that apoptosis plays in the function of fistula.

- Our results suggest that apoptosis may play a role in fistula failure, but we cannot conclude that apoptosis is the main cause of fistula failure. Further research is required to investigate this point.

## 8. SAŽETAK (ABSTRACT IN CROATIAN)


### APOPTOZA U STIJENCI NATIVNE VENE U NEUSPJEHU ARTERIOVENSKIH FISTULA ZA HEMODIJALIZU

U skladu sa rastućim interesom za proučavanje apoptoze u krvnim žilama, ovo istraživanje je jedno od prvih koje procjenjuje apoptozu u nativnim venama namijenjenim za arteriovenske fistule (AVF) u hemodijalizi. Cilj ovog istraživanja bio je procijeniti apoptozu u prethodno probušenim nativnim venama (istraživana skupina) u usporedbi s neprobušenim nativnim venama (kontrolna skupina) u bolesnika koji su podvrgnuti kirurškom postupku za AVF kao pristupu za dijalizu. Uzorci cefalične vene dobiveni su od 60 bolesnika prije postavljanja AVF. Polovica uzoraka bila je iz prethodno probušenih, a polovica iz neprobušenih vena. Iz distalnog dijela cefalične vene izrezan je dio duljine 1-cm, podijeljen u dva dijela duž uzdužne osi i pripremljen za imunohistokemijsku analizu. Imunohistokemijska metoda i kvantifikacija signala se koristila za procjenu proteinske ekspresije Bax, p53, kaspaze 3 i Bcl-2.

Uzorci vena iz istraživane skupine s prethodno probušenim venama pokazali su značajno povećani izražaj kaspaze 3 i Bax u usporedbi s kontrolnom skupinom ($p < 0.01$). Izražaj Bcl-2 u istraživanoj skupini bio je značajno smanjen u odnosu na kontrolnu skupinu ($p < 0,01$). Izražaj p53 nije pokazao značajnu razliku između dviju skupina ($p = 0,791$). Otkrivena je statistički značajna razlika u neuspjehu fistule između istraživane i kontrolne skupine (26,7% u odnosu na 6,7%, $p = 0,038$). Veza koju smo otkrili između prethodno probušene vene i apoptoze ukazuje na ulogu venepunkcije u razvoju apoptoze. Bolesnici s povećanom apoptozom u cefaličnoj veni pokazali su povećan neuspjeh fistule, pa je to saznanje od značaja za poboljšanje postupka izvedbe AVF.

## 9. ABSTRACT

In line with the growing interest for studying apoptosis in blood vessels, this study is one of the first to assess the apoptosis in native veins used for arteriovenous fistulas (AVF) done for hemodialysis. The aim of this study was to evaluate apoptosis in previously punctured native veins (study groupy) compared with not punctured native veins (controls) in patients who undergo a surgical procedure for AVF as dialysis access. Cephalic vein specimens were obtained from 60 patients before the placement of AVF. Half of the specimens were from the previously punctured and half from non-punctured veins. A 1-cm long segment was excised from distal part of the cephalic vein, divided into two portions along longitudinal axis and prepared for immunohistochemical analysis. Immunohistochemical assessment and quantification of signals was used to evaluate the expression of Bax, p53, caspase 3 and Bcl-2.

Vein specimens from the study group with previously punctured veins showed significantly increased caspase 3 and Bax expression, compared with the control group ($p<0.01$). Bcl-2 expression in the study group was significantly decreased compared with the control group ($p<0.01$). p53 showed no significant differences between the two groups ($p=0.791$). There were statistically significant differences in fistula failure between the study group and control group (26.7% *versus* 6.7%, $p=0.038$). The association we have found between previously punctured veins and apoptosis indicates the role that venipuncture may play in the development of apoptosis. Patients with increased apoptosis showed an increased fistula failure, which is of importance for the improvement of the AVF procedure itself.

**Keywords: native vein, apoptosis, venipuncture, hemodialysis, arteriovenous fistula, fistula failure**

## 10. REFERENCES


1. Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol 2007; 35:495-516.
2. Hayakawa Y, Takemura G, Misao J et al. Apoptosis and overexpression of bax protein and bax mRNA in smooth muscle cells within intimal hyperplasia of human radial arteries: analysis with arteriovenous fistulas used for hemodialysis. Arterioscler Thromb Vasc Biol 1999; 19:2066-77.
3. Sanz AB, Santamaría B, Ruiz-Ortega M, Egido J, Ortiz A. Mechanisms of renal apoptosis in health and disease. J Am Soc Nephrol 2008; 19:1634-42.
4. Björkerud S, Björkerud B. Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. Am J Pathol 1996; 149:367-80.
5. Krijnen PA, Nijmeijer R, Meijer CJ et al. Apoptosis in myocardial ischaemia and infarction. J Clin Pathol 2002; 55:801-11.
6. Czerski L, Nunez G. Apoptosome formation and caspase activation: is it different in the heart? J Mol Cell Cardiol 2014; 37:643-652.
7. Ferencić Z. Apoptosis in toxicologic pathology. Acta Med Croatica 2009; 63:33-6.
8. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes Cells 1998; 3:697-707.
9. Tsujimoto Y, Shimizu S. Bcl-2 family: life-or-death switch. FEBS Lett 2000;466(1):6-10.
10. Robbins and Cotran. Apoptosis. In: Robbins and Cotran. Pathologic Basis of Disease. Elsevier Saunders, 8th edn, Philadelphia 2010, pp39-46.

11. Mallat Z, Tedgui A. Apoptosis in the vasculature: mechanisms and functional importance. Br J Pharmacol 2000; 130:947-962.
12. Majno G, Joris I. Apoptosis, oncosis, and necrosis. An overview of cell death. Am J Pathol 1995; 146:3-15.
13. Saraste A. Morphologic criteria and detection of apoptosis. Herz 1999; 24:189-95.
14. Kovačević M, Simić O, Jonjić N, Stifter S. Apoptosis and cardiopulmonary bypass. J Card Surg 2007; 22:129-34.
15. Robbins and Cotran. Apoptosis. In: Robbins and Cotran. Pathologic Basis of disease. Saunders, 8th edn, 2010, p27.
16. Kumar V, Abbas A, Fausto N. Cellular Adaptations, Cell Injury, and Cell Death. In: Kumar V, Abbas A, Fausto N. Robbins and Cotran Pathologic Basis of Disease. Elsevier Saunders, Philadelphia 2010, pp13-22.
17. Leszczynski D, Zhao Y, Luokkamaki M, Foeght M. Apoptosis of vascular smooth muscle cells. Protein kinase C and oncoprotein Bcl-2 are involved in regulation of apoptosis in non-transformed rat vascular smooth muscle cells. Am J Pathol 1995; 145:1265-70.
18. Antonsson B, Martinou JC. The Bcl-2 protein family. Exp Cell Res 2000; 256:50–57.
19. Bialik Sh, Cryns VL, Drincic A, Miyata S et al. The mitochondrial apoptotic pathway is activated by serum and glucose deprivation in cardiac myocytes. Circ Res 1999; 85:403-414.
20. Wu ZK, Laurikka J, Saraste A et al. Cardiomyocyte apoptosis and ischemic preconditioning in open heart operations. Ann Thorac Surg 2003; 76:528-34.

21. Pećina-Slaus N. Genetic and molecular insights into apoptosis. Acta Med Croatica 2009; 63:13-9.

22. Raffetto J. Chronic venous insufficiency:molecular abnormalities and ulcer formation. In: Bergan J, Bunke-Paquette. The vein book. Oxford University Press, New York 2014. pp58-67.

23. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 2000; 45:528–537.

24. Kumar V, Abbas A, Fausto N, Aster J. Cellular Responses to Stress and Toxic Insults: Adaptation, Injury, and Death. In: Kumar V, Abbas A, Fausto N. Robbins and Cotran Pathologic Basis of Disease. Elsevier Saunders, Philadelphia 2010. pp39-46.

25. Belicza M. Evaluation of morphologically determined apoptotic index. Acta Med Croatica 2009; 63:3-12.

26. Ducasse E, Giannakakis K, Speziale F et al. Association of primary varicose veins with disregulated vein wall apoptosis. Eur J Vasc Endovasc Surg 2008; 35:224-229.

27. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995; 80:293-299.

28. Kruse JPh, Gu W. Modes of p53 regulation. Cell 2009; 137:609–622.

29. Krušlin B. Apoptosis in pathologic prostatic processes. Acta Med Croatica 2009; 63:49-52.

30. Urbanek T, Skop B, Wiaderkiewicz R et al. Smooth muscle cell apoptosis in primary varicose veins. Eur J Vasc Endovasc Surg 2004; 28:600-611.

31. Urbanek T, Skop B, Ziaja K et al. Sapheno-femoral junction pathology, molecular mechanism of saphenous vein incompetence. Clin Appl Thromb Hemost 2004; 10:311-321.
32. Hawes D, Shi ShR, Dabbs D, Taylor C, Cote R. Immunohistochemistry. In: Weidner, Cote, Suster, Weiss. Modern Surgical Pathology. Elsevier Saunders, Philadelphia 2009. pp48-52.
33. Filis K, Kavantzas N, Dalainas I et al. Evaluation of apoptosis in varicose vein disease complicated by superficial vein thrombosis. Vasa 2014; 43:252-9.
34. McIlwain DR, Berger Th, Mak TW. Caspase Functions in Cell Death and Disease. Cold Spring Harb Perspect Biol 2015; 5: a008656
35. Porter A, Janicke R. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999; 6:99-104.
36. Loreto C, Almeida LE, Migliore MR, Caltabiano M, Leonardi R. TRAIL, DR5 and caspase 3-dependent apoptosis in vessels of diseased human temporomandibular joint disc. An immunohistochemical study. Eur J Histochem 2010; 54:40.
37. S.S.M. Rensen, P.A.F.M. Doevendans, G.J.J.M. van Eys. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Neth Heart J 2007; 15:100–108.
38. Kovačević M, Jonjić N, Stalekar H, Zaputovič L, Stifter S, Vitezic D. Apoptotic cell death and rupture of abdominal aortic aneurysm. Med Hypotheses 2010; 74:908-10.
39. Clarke M, Bennett M, Littlewood T. Cell death in the cardiovascular system. Heart 2007; 93:659–664.
40. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801-809.

41. Swirski F, Nahrendorf M. Do vascular smooth muscle cells differentiate to macrophages in atherosclerotic lesions. Circ Res 2014; 115:605-606.
42. Bartels C, Malisius R, Sayk F et al. Accelerated smooth muscle cell apoptosis in occluded aorto-coronary saphenous vein graft. Int J Angiol 2001; 10:237-240.
43. Isner JM, Kearney M, Bortman S, Passeri J. Apoptosis in human atherosclerosis and restenosis. Circulation 1995; 91:2703-2711.
44. Mayr U, Mayr M, Li C et al. Loss of p53 accelerates neointimal lesions of vein bypass grafts in mice. Circ Res 2002; 90:197-204.
45. Langer S, Kokozidou M, Heiss C et al. Chronic kidney disease aggravates arteriovenous fistula damage in rats. Kidney Int 2010; 78:1312-1321.
46. Urbanek T. Apoptosis and cell cycle regulation in the vein wall-new elements related to varicose vein occurrence. Phlebolymphology 2005; 49:403-410.
47. Bastos AN, Alves MM, Monte-Alto-Costa A et al. α-smooth muscle actin, fibrillin-1, apoptosis and proliferation detection in primary varicose lower limb veins of women. Int Angiol 2011; 30:262-267.
48. Filis K, Kavantzasb N, Isopoulosa T et al. Increased vein wall apoptosis in varicose vein disease is related to venous hypertension. Eur J Vasc Endovasc Surg 2011; 41:533-539.
49. Bujan J, Jimenez-Cossio JA, Jurado F et al. Evaluation of the smooth muscle cell component and apoptosis in the varicose vein wall. Histopathol 2000; 15:745–752.
50. Simovart HE, Aunapuu M, Lieberg J, Roosaar P, Arend A. Age-related changes in apoptosis and expressions of intercellular adhesion molecule-1 and vascular endothelial growth factor receptor type 2 in the wall of varicose veins. Int Angiol 2010; 29:507-513.

51. Simovart HE, Arend A, Lieberg J, Aunapuu M. Associations of NF-kappaB and Bax with apoptosis in varicose veins of women of different age groups. Int J Vasc Med 2011; 1-7. Article ID 639720, 7 pages http://dx.doi.org/10.1155/2011/639720.
52. Szczech L, Harmon W, Hostetter Th et al. World Kidney Day 2009: Problems and challenges in the emerging epidemic of kidney disease. J Am Soc Nephrol 2009; 20:453-455.
53. Denker B, Chertow G, Owen W. Hemodialysis. In: Brenner B Ed. The Kidney. W.B. Saunders Company, Philadelphia 2000. pp373-2457.
54. Langer S, Paulus N, Koeppel TA et al. Cardiovascular remodelling during arteriovenous fistula maturation in a rodent uremia model. J Vasc Access 2011; 12(3):215-23.
55. Allon M, Robbin ML. Increasing arteriovenous fistulas in hemodialysis patients: problems and solutions. Kidney Int 2002; 62:1109-1124.
56. Wylie E, Stoney R, Ehrenfeld W, Effeney D. Hemodialysis Access. In: Wylie E, Stoney R, Ehrenfeld W, Effeney D. Manual of Vascular Surgery. Springer-Verlag, New York 1986. pp312-320.
57. Gelabert HA, Freischlag JA. Hemodialysis access. In: Rutherford R. Vascular Surgery. W.B. Saunders Company, Philadelphia 2000. pp1466-1476.
58. Davidson I. The end stage renal disease patient as related to dialysis. In: Access for Dialysis: Surgical and Radiologic Procedures, 2nd Edition. Landes Bioscience, Texas 2002. pp1-10.
59. Musial K, Zwolinska D. New markers of apoptosis in children on chronic dialysis. Apoptosis 2013; 18:77–84.

60. Atamaniuk J, Kopecky C, Skoupy S et al.Apoptotic cell-free DNA promotes inflammation in haemodialysis patients. Nephrol Dial Transplant 2012; 27:902-905.
61. Carracedo J, Ramirez R, Madueno JA et al. Cell apoptosis and hemodialysis-induced inflammation. Kidney Int 2002; 61:89-93.
62. Galli F, Ghibelli L, Buoncristiani U et al. Mononuclear leukocyte apoptosis in haemodialysis patients: the role of cell thiols and vitamin E. Nephrol Dial Transplant 2003; 18:1592-1600.
63. Boccellino M, La Porta R, Coppola M et al. Peritoneal dialysis fluid activates calcium signaling and apoptosis in mesothelial cells. Apoptosis 2013; 18:43-56.
64. Wu CC, Liao TN, Lu KC et al. Apoptotic markers on lymphocytes and monocytes are unchanged during single hemodialysis sessions using either regenerated cellulose or polysulfone membranes. Clin Nephrol 2005; 64:198-204.
65. Majewska E, Baj Z, Sulowska Z, Rysz J, Luciak M. Effects of uraemia and haemodialysis on neutrophil apoptosis and expression of apoptosis-related proteins. Nephrol Dial Transplant 2003; 18:2582-2588.
66. Bergamini TM, Taber SW, Hoch JR. Long-term venous access. In: Rutherford R. Vascular Surgery. W.B. Saunders Company, Philadelphia 2000. pp1487-1492.
67. Pansky B. Upper extremity. In: Pansky B, ed. Review of gross anatomy. McGraw Hill, 6th edn 1996; pp231-324.
68. Wellen J, Shenoy S. Ultrasound in vascular access. In: Vascular Access: Principles and Practise. Wilson SE, ed. Philadelphia, PA: Lippincott Williams&Wilkins, 5th edn, 2009; pp234-242.

69. Shenoy S. Surgical anatomy of upper arm: what is needed for AVF planing. J Vasc Access 2009; 10:223-232.

70. Chin EE, Zimmerman PT, Grant EG. Sonographic evaluation of upper extremity deep venous thrombosis. J Ultrasound Med 2005; 24:829-838.

71. Brkljačić B. Doplerski pregled perifernih arterija. In: Brkljačić B. Vaskularni ultrazvuk. Medicinska naklada, Zagreb 2010; pp106-109.

72. Brkljačić B. Doplerski pregled perifernih vena. In: Brkljačić B. Vaskularni ultrazvuk. Medicinska naklada, Zagreb 2010; pp147-151.

73. Bonnuchi D, Cappelli G, Albertazzi A. Which is the preferred vascular access in diabetic patients. Nephrol Dial Transplant 2002; 17:20-22.

74. Chan Ph. Vascular smooth muscle cells: structure and function. In: Fitridge R, Thomson M. Mechanism of Vascular Disease. Cambridge University Press, New York 2006;14-22.

75. Moore Kl. Upper limb. In: Moore KL, Dalley AF et al. Clinically oriented anatomy. Lippincott, Williams and Wilkins, 5$^{th}$edn, Philadelphia 2006; pp726-88.

76. Mitchell R, Schoen F. Blood Vessels. In: Kumar V, Abbas A, Fausto N. Robbins and Cotran Pathologic Basis of Disease. Elsevier Saunders, Philadelphia 2010; pp487-489.

77. Cooley D, Wukasch D. Dialysis Shunts. In: Cooley D, Wukasch D. Techniques in vascular surgery. W.B. Saunders Company, Philadelphia 1979; pp106-112.

78. Ascher E, Gade P, Hingorani A et al. Changes in the practice of angioaccess surgery: impact of dialysis outcome and qualitative initiative recommendations. J Vasc Surg 2000; 31:84-92.

79. Hamilton HC, Foxcroft Dr. Central venous access sites for the prevention of venous thrombosis, stenosis and infection in patients requiring long-term intravenous therapy. Cochrane Database Syst Rev 2007; (3):CD004084.

80. Konner K, Nonnast-Daniel B, Ritz E. The arteriovenous fistula. J Am Soc Nephrol 2003; 14:1669-1680.

81. Beathard GA, Settle S, Shields M. Salvage of the nonfunctioning arteriovenous fistula. Am J Kidney Dis 1999; 33:910-916.

82. Dixon BS, Novak L, Fangman J. Hemodialysis vascular access survival: upper-arm native arteriovenous fistula. Am J Kidney Dis 2002; pp39:92.

83. Derakhshanfar A, Gholyat M, Njavesh A, Bahiraii S. Assessment of frequency of complications of arteriovenous fistula in patients on dialysis: a two-year single center study from Iran. Saudi J Kidney Dis Transpl 2009; 20:872-875.

84. Moncef G. Surgical revision of failing or thrombosed native arteriovenous fistulas: A single center experience. Saudi J Kidney Dis Transpl 2010; 21:258-261.

85. Sidawy AN, Gray R, Besarab A et al. Recommended standards for reports dealing with arteriovenous hemodialysis accesses. J Vasc Surg 2002; 35:603-610.

86. Smith G, Gohil R, Chetter I. Factors affecting the patency of arteriovenous fistulas for dialysis access. J Vasc Surg 2012; 55:849-855.

87. Deneuville M. Infection of PTFE grafts used to create arteriovenous fistulas for hemodialysis access. Ann Vasc Surg 2000; 14:473-479.

88. O'Hare AM, Bertenthal D, Walter LC et al. When to refer patients with chronic kidney disease for vascular access surgery: Should age be a consideration? Kidney Int 2007; 71:555-561.

89. Lin PH, Bush RL, Nguyen L et al. Anastomotic strategies to improve hemodialysis access patency. Eur J Vasc Endovasc Surg 2005; 39:135-42.

90. Karakayall F, Sevmis S, Basaran C et al. Relationship of preoperative venous and arterial imaging findings to outcomes of brachio-basilic transposition fistulae for hemodialysis: a prospective clinical study. Eur J Vasc Surg 2008; 35:208-213.

91. Mizobuchi M, Towler D, Slatopolsky E. Vascular calcification: the killer of patients with chronic kidney disease. J Am Soc Nephrol 2009; 20:1453-1464.

92. Misanović V, Jonuzi F, Anić D, Halimić M, Rahmanović S. Central venous catheter as vascular approach for hemodialysis - our experiences. Mater Sociomed 2015; 27:112-113.

93. Premužić V, Tomašević B, Eržen G et al. Temporary and permanent central venous catheters for hemodialysis. Acta Med Croatica 2014; 68:167-174.

94. Sedlacek M, Teodorescu V, Falk A et al. Hemodialysis access placement with preoperative noninvasive vascular mapping: Comparison between patients with and without diabetes. Am J Kidney Dis 2001; 38:560−564.

95. Nikeghbalian S, Bananzadeh A, Yarmohammadi H. Difficult vascular access in patients with end-stage renal failure. Transplant Proc 2006; 38:1265-1266.

96. Pantea S, Bengulescu I. Smooth loop arterio-venous fistula. Chirurgia(Bucur) 2014; 109:678-681.

97. Ferrari G, Talassi E, Baraldi C et al. A good vascular access allows an effective treatment. G Ital Nefrol 2005; 22:60-69.

98. Roy-Chaudhury P, Sukhatme VP, Cheung AK. Hemodialysis vascular access dysfunction: a cellular and molecular viewpoint. J Am Soc Nephrol 2006; 17:1112-1127.

99. Bia D, Zócalo Y, Armentano R et al. Vascular access for haemodyalisis. Comparative analysis of the mechanical behaviour of native vessels and prosthesis. Nefrologia 2006; 26:587-593.

100. Ferrari G, Talassi E, Baraldi C, Lambertini D, Tarchini R. Vascular access validity and treatment efficiency in hemodialysis. G Ital Nefrol 2003; 20:22-29.

101. Ferring M, Claridge M, Smith SA, Wilmink T. Routine preoperative vascular ultrasound improves patency and use of arteriovenous fistulas for hemodialysis: a randomized trial. Clin J Am Soc Nephrol 2010; 5:2236-2244.

102. Lockhart ME, Robbin ML, Fineberg NS et al. Cephalic vein measurement before forearm fistula creation: does use of a tourniquet to meet the venous diameter threshold increase the number of usable fistulas? J Ultrasound Med 2006; 25:1541-1545.

103. Banerjee S. Fistula maturation and patency for successful dialysis. Dialysis Transplant 2009; 38:442.

104. Cifarelli M. Graft or CVC? A prosthetic graft is the better choice. G Ital Nefrol 2009; 26:148-153.

105. Lacson E, Wang W, Lazarus J, Hakim R. Change in vascular access and hospitalization risk in long term hemodialysis patients. Clin J Am Soc Nephrol 2010; 5:1996–2003.

106. Peck MK, Dusserre N, Zagalski K et al. New biological solutions for hemodialysis access. J Vasc Access 2011; 12:185-192.

107. Davidson I. The end stage renal disease patient as related to dialysis. In: Access for Dialysis: Surgical and Radiologic Procedures, 2nd Edition. Landes Bioscience, Texas 2002; pp1-10.

108. Santoro D, Benedetto F, Mondello P et al. Vascular access for hemodialysis: current perspectives. Int J Nephrol Ren Dis 2014; 7:281-294.

109. Rooijens P.P.G.M, Tordoir J.H.M, Stijnen T et al. Radiocephalic wrist arteriovenous fistula for hemodialysis: Meta-analysis indicates a high primary failure rate. Eur J Vasc Endovasc Surg 2004; 28: 583–589.

110. Twine CP, Haidermota M, Woolgar JD, Gibbons CP, Davies CG. A scoring system (DISTAL) for predicting failure of snuffbox arteriovenous fistulas. Eur J Vasc Surg 2012; 44:88-91.

111. Ferring M, Henderson J, Wilmink A, Smith S. Vascular ultrasound for the pre-operative evaluation prior to arteriovenous fistula formation for haemodialysis: review of the evidence. Nephrol Dial Transplant 2008; 23:1809-1815.

112. Ashraf T, Panhwar Z, Habib S, Memon MA, Shamsi F, Arif J. Size of radial and ulnar artery in local population. J Pak Med Assoc 2010; 60:817-819.

113. Matoussevitch V, Konner K, Gawenda M et al. A modified approach of proximalization of arterial inflow technique for hand ischaemia in patients with matured basilic and cephalic veins. Eur J Vasc Surg 2014; 48:472-476.

114. Leaf DA, MacRae HS, Grant E, Kraut J. Isometric exercise increases the size of forearm veins in patients with chronic renal failure. Am J Med Sci 2003; 325:115-119.

115. Asif A, Roy-Chaudhury P, Beathard GA. Early arteriovenous fistula failure: a logical proposal for when and how to intervene. Clin J Am Soc Nephrol 2006; 1:332-339.

116. Beathard GA. An algorithm for the physical examination of early fistula failure. Semin Dial 2005; 18:331.

117. Woo K, Goldman DP, Romley JA. Early failure of dialysis access among the elderly in the era of fistula first. Clin J Am Soc Nephrol 2015; 10:1791-1798.

118. Beathard GA. Strategy for maximizing the use of arteriovenous fistulae. Semin Dial 2000; 13:291-296.

119. Roy-Chaudhury P, Kruska L. Future directions for vascular access for hemodialysis. Semin Dial 2015; 28:35-40.

120. Rushing J. Care for a patient's vascular access for haemodialysis. Nurs Manage 2010; 41:47.

121. Kheda MF, Brenner LE, Patel MJ et al. Influence of arterial elasticity and vessel dilatation on arteriovenous fistula maturation: a prospective cohort study. Nephrol Dial Transplant 2010; 25:525–531.

122. Ku YM, Kim YO, Kim JI et al. Ultrasonographic measurement of intima-media thickness of radial artery in pre-dialysis uraemic patients: comparison with histological examination. Nephrol Dial Transplant 2006; 21:715-20.

123. Rodrigues LT, Pengloan J, Rodrigue H et al. Treatment of failed native arteriovenous fistulas for hemodialysis by interventional radiology. Kidney Int 2000; 57:1124-1140.

124. Kavurma MM, Bhindi R, Lowe HC, Chesterman C, Khachigian LM. Vessel wall apoptosis and atherosclerotic plaque instability. J Thromb Haemost 2005; 3:465-472.

125. Ducasse E, Giannakakis K, Chevalier J et al. Dysregulated apoptosis in primary varicose veins. Eur J Vasc Endovasc Surg 2005; 29:316-323.

126. Lim CS, Davies AH. Pathogenesis of primary varicose veins. Br J Surg 2009; 96:1231-1242.

127. Raffetto JD, Khalil RA. Mechanisms of varicose vein formation: valve dysfunction and wall dilation. Phlebology 2008; 23:85-98.

128. Valen G. The basic biology of apoptosis and its implications for cardiac function and viability. Ann Thorac Surg 2003; 75:656-60.

129. Ascher E, Jacob T, HingoraniA et al. Programmed cell death (apoptosis) and its role in the pathogenesis of lower extremity varicose veins. Ann Vasc Surg 2000; 14:24-30.

130. Ducasse E, Fleurisse L, Vernier G, Speziale F, Fiorani P, Puppinck P, Creusy C. Interposition vein cuff and intimal hyperplasia: an experimental study. Eur J Vasc Endovasc Surg 2004; 27:617-621.

131. Wong Pn, Fung TT, Mak SK et al. Hepatitis B virus infection in dialysis patients. J Gastroenterol Hepatol 2005; 20:1641-1651.

132. Planken NR, Kenter HX, Kessel GA, Hocks PA, Leiner T, Tordoir J. Forearm cephalic vein cross-sectional area changes at incremental congestion pressures: Towards a standardized and reproducible vein mapping protocol. J Vasc Surg 2006; 44:353-358.

133. Van der Veer S, Ravani P, Coentrao L, Fluck R, Kleophas W, Labriola L. Barriers to adopting a fistula-first policy in Europe: an international survey among national experts. J Vasc Access 2015; 16:113-119.

134. Deblois D, Tea BS, Beaudry D, Hamet P. Regulation of therapeutic apoptosis: a potential target in controlling hypertensive organ damage. Can J Physiol Pharmacol 2005; 83:29-41.

135. Haunstetter A, Izumob S. Future perspectives and potential implications of cardiac myocyte apoptosis. Cardiovasc Res 2000; 45:795–801.

136. Kockx MM, Cambier BA, Bortier HE et al. Foam cell replication and smooth muscle cell apoptosis in human saphenous vein grafts. Histopathol 1994; 25:365-371.

137. Ascher E, Jacob T, Hingorani A, Tsemekhin B, Gunduz Y. Expression of molecular mediators of apoptosis and their role in the pathogenesis of lower-extremity varicose veins. J Vasc Surg 2001; 33:1080-1086.

138. Hammond EM, Giaccia AJ. The role of p53 in hypoxia-induced apoptosis. Biochem Biophys Res Commun 2005; 331:718-725.

139. Jacob T, Hingorani A, Ascher E. Overexpression of transforming growth-factor-beta 1 correlates with increased synthesis of nitric oxide synthase in varicose veins. J Vasc Surg 2005; 41:523-530.

## 11. CURRICULUM VITAE

I was born on June 02, 1974, in Prishtina, Kosovo, where I completed primary school and gymnasium. In 2000, I graduated from the School of Medicine, University of Prishtina.

I completed specialization in Vascular Surgery, at the University Clinical Center of Kosovo (UCCK), Prishtina, from 2001 to 2007.

I defended Master's thesis at the School of Medicine, University of Prishtina, in 2006.

In 2009, I attended the continuing professional education at the University Clinic of Surgery, in Mannheim, University of Heidelberg, Germany. I attended several postgraduate courses: a CME postgraduate course in Intensive Education in Ultrasound of Abdomen and Large Blood Vessels, Kosovo, in 2010; a CME postgraduate course in Endovascular Surgery, in Zagreb, Croatia, in 2011; a CME postgraduate course in Vascular Ultrasound, in Opatija, Croatia, in 2014.

I am a member of the European Society for Vascular Surgery (ESVS).

I am the author of 5 papers in peer reviewed journals, and of 3 papers in national journals. I had oral and poster presentation at national and international conferences.

I have been a teaching assistant in Surgery, at the School of Medicine, University of Prishtina, since 2007. I have been working as a vascular surgeon at the Clinic of Vascular Surgery, University Clinical Center of Kosovo, in Prishtina, since 2007.

I am fluent in Albanian, English and Croatian language.

I am married, and a mother of two children.