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R. A. Moskalenko, A. M. Romaniuk

Pathological biomineralization of soft tissues

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Reviewers:
M. V. Pogorelov – Doctor of Medical Sciences, Professor, Chief Researcher of the Center for Collective Use of Equipment of the Medical Institute of SSU;
S. M. Danilchenko – Candidate of Physical and Mathematical Sciences, Head of the Department of Radiation Biophysics of the Institute of Applied Physics of the National Academy of Sciences of Ukraine

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The monograph presents the results of the study of pathological soft tissue biomineralization in atherosclerosis, dystrophy, inflammation, benign and malignant tumor growth on the example of atherosclerosis of the aorta and aortic valves, papillary and follicular thyroid cancer, benign prostatic hyperplasia, diseases of the gallbladder, pancreas, salivary and breast glands, eyes. The publication is intended for researchers of medical, biological and natural sciences, biophysics specialists, pathologists, oncologists, radiologists, surgeons, as well as for clinical residents, interns and senior students of medical universities.

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CONTENT

PREFACE

Recently, doctors have been increasingly seeing biomineralization in their practice. It is observed primarily in patients with impaired metabolism. However, sometimes the manifestations of increased biomineralization of various organs and tissues in patients are quite unexpected for the clinician. This causes complexity in the treatment, prognosis of the disease. As a result, there is a need for a thorough study and understanding of this pathological process. Thanks to the introduction of modern morphological, physico-technical methods of research in medicine, the diagnostics of the process of tissue biomineralization in various pathological processes have significantly improved. Today, significant progress has been made in the study and understanding of this biological phenomenon in living and dead tissues in various pathologies, but many questions remain. One of them – biomineralization – is a positive or negative process in the human body. There are enough publications in the literature that clarify biomineralization from different positions for a living organism. It is our work that addresses these issues, which will allow us to open the veil of a complex living organism that is struggling with various pathological processes, especially with tumor growth. Our work is based on fundamental studies of biomineralization in the laboratories of Sumy State University (Ukraine) and Umeå University (Sweden). We hope that the results of our research will be useful for all specialists who are interested in the study of complex bioprocesses in living organisms.

Head of the Department of Pathology,
Sumy State University,
Doctor of Medical Sciences, Prof. A. M. Romaniuk

LIST OF SYMBOLS AND ABBREVIATIONS

AV – aortal valve.
AFM – atomic force microscopy.
BHP – benign hyperplasia of prostate.
CA – corpora amylacea or amyloid bodies in prostate.
Casp-3 – caspase 3.
ChCCh – chronical calculous cholecystite.
CIC – circulating inflammatory cells.
CAV – calcified aortic valves.
CHD – coronary hearth disease.
GB – gallbladder.
GC – gallbladder carcinoma.
EC – endothelial cells.
IHC – immunihistochemistry.
IRS – infrared spectroscopy.
JCPDS – Joint Committee on Powder Diffraction Standards
MPO – myeloperoxidase.
MTT– malignant thyroid tumor.
MV – microvesicles.
OPG – osteoprotegerine.
OPN – osteopontine.
VC – vascular calcification.
PB – psammoma bodies.
PPi - pyrophosphate mineralization inhibitor.
PBM – pathological biomineralization.
PBG – porcelain gallbladder.
PTC – papillary thyroid carcinoma.
SEM – scanning electronic microscopy.
TEM – transparent electronic microscopy.
TNSALP - tissue nonspecific alkaline phosphatase.
TG – thyroid carcinoma.
VSMC – vascular smooth muscle cell.
XRD – X-ray diffraction.

INTRODUCTION

In recent decades, the interest in the study of pathological biomineralization in the human body has increased significantly. This is due to a significant increase in the incidence of pathology associated with this phenomenon [1, 2, 3].

Biomineralization is the process of formation of minerals by living organisms under the conditions of formation and growth of biominerals from a supersaturated aqueous solution in an organic matrix having a cellular origin [4]. In living organisms, biomineralization develops into three types: silicon, carbonate, and phosphate. Today, about 300 biominerals are known [5].

Biophysical, biochemical and pathogenetic features of biomineralization under conditions of internal and external adverse factors have not been sufficiently studied. Data from scientific literature regarding the influence of exo- and endogenous factors on the initiation and development of pathological biomineralization (PBM) processes are controversial. Regeneration and repair possibilities of body tissues under conditions of development of PBM processes remain a little researched question. It is important to find ways to treat and prevent pathological biomineralization.

A considerable amount of new scientific data and facts about pathological biomineralization have appeared in the literature. Due to the rapid development of molecular biology, biochemistry, pathological physiology and morphology, the introduction of new research methods into medical practice, the view on the traditional division of pathological mineralization or mineral dystrophy into metastatic, dystrophic and metabolic is changing. The active nature of this process and the similarity of mineral dystrophy to the physiological processes of biomineralization in solid tissues of the body become evident.

Therefore, despite a large amount of new knowledge about the mechanisms and nature of pathological biomineralization of soft tissues, there is no clear answer as to the direction, clinical, morphological and prognostic significance of it under different pathological processes, such as benign and malignant tumor growth, chronic inflammation, dystrophy and atherosclerosis.

This research was performed as part of the investigation topics of Pathology Department of Sumy State University № 0117U003937 "Development of diagnostic method of the reproductive system tumors using cellular adhesion molecules of the cancer-embryonic antigen" and № 0118U003570 "Efficiency of liquid biopsy and tissue biopsy in the malignant tumors diagnostic and treatment".

1 SOFT TISSUE BIOMINERALIZATION

1.1 Brief historical sketch of the development of biomineralogy

The development of biomineralogy as a science is divided into two main stages: the period of formation of science, which lasted until the middle of the twentieth century, and the period of existence as a separate branch of scientific knowledge [6].

In the first period of development of biomineralogy there was an accumulation of data on the structure, composition, genesis of biominerals. Biomineralization has been a process for over one billion years. Formation of the first biominerals began in the Precambrian and continues to this day, accompanying the evolutionary development of living organisms [5]. One of the first modern scientists to pay attention to the problems of biomineralization was academician V.I. Vernadsky in the 20's of the twentieth century. On the territory of the former USSR, the term "biomineralogy" was first used by A. Korago in 1976 in a work devoted to the study of river pearls [6]. The first work, where the term "biomineralogy" was used in the name, was published in Kyiv, a monograph by B. I. Srebnodolsky "Biological mineralogy" in 1983, which was devoted to mineralization processes in the body of animals [7]. A significant event in the development of this science was the publication of the fundamental work "Introduction to Biomineralogy" in 1992 by A. Korago [6]. Now biomineralogy is a field of scientific knowledge that is on the border of various sciences and is rapidly developing. This issue is covered by a number of foreign specialized journals and conferences (Calcified Tissue Int, Eur J of Mineralization, Bone, Journal of Crystal Growth).

The process of biominerals research is complicated by the complex interaction of the organic and mineral components

that make up them. The organic and mineral components of biominerals are inextricably linked and interact under certain laws, which is why the term "organic-mineral aggregates" (OMA) has been consolidated [8]. Biominerals or OMA of the human body are divided into physiological and pathological.

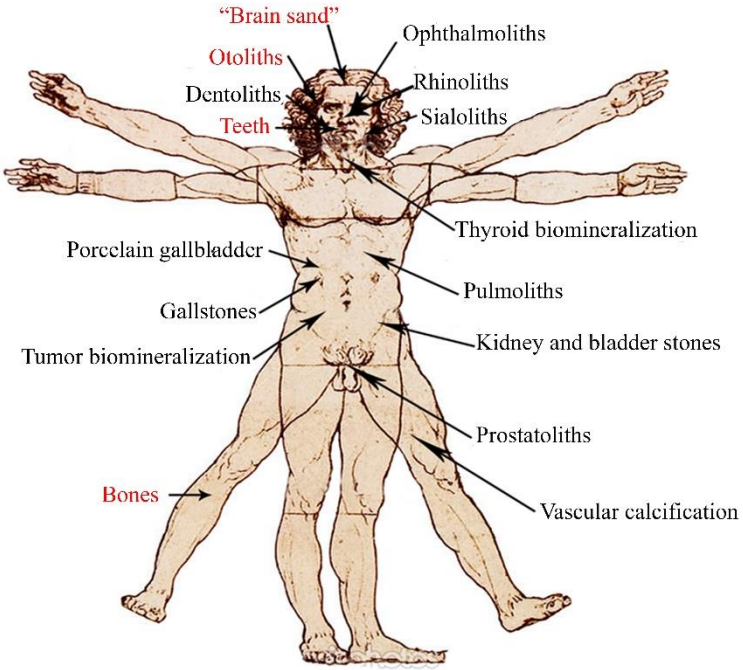

Figure 1.1 – Biomineralization in the human body

1.2 Physiological biomineralization

In the body of a healthy person, physiological biomineralization is limited by the solid tissues of the oral cavity, the skeleton, the equilibrium organ (otoliths), and the brain epiphysis tissue (the deposition of "brain sand").

Teeth. Teeth include mineralized tissues such as enamel, dentin and cement. In the human body, the most mineralized tissue is tooth enamel, the mineralization of which is 95-97 % of

its weight [9, 10]. Dentin, which forms the major part of the solid tissues of the tooth, has 72 % of inorganic substances, the major part of which corresponds to the hydroxyapatite mineral [11]. Cement wraps around the root of the tooth and is involved in attaching the tooth to the alveolar bone. It is slightly softer than dentin and is composed of
45-50 % inorganic substances (hydroxyapatite). In addition to hydroxyapatite, the basis of the mineral tissues of the teeth is octacalphosphate and in small quantities other forms of apatites – carboxyapatite, chlorapatite, strontium apatite and fluorapatite [12].

Bones. There are approximately 270 bones in the human body [13]. Bone is by nature a relatively lightweight and durable composite material containing inorganic and organic parts. The bone mineral component reaches 60-70 % of the weight and is also mainly composed of hydroxyapatite [13, 14]. The organic part of the bone is represented mainly by collagen, an elastic protein that provides resistance to fractures [14]. In addition to collagen, which are structural proteins, a significant proportion of bone proteins are regulators of the biomineralization process: osteopontin, osteonectin, osteocalcin, bone sialoprotein, and phosphor dentin [15]. In bones, mineralization begins with a heterogeneous solution of calcium and phosphorus ions. Mineral nucleates are generated in the empty spaces between the collagen fibers in the form of a thin layer of hydroxyapatite, which grows to fill the available space. However, the mechanism of deposition of minerals in the organic part of the bone even today is not fully understood and studied [15].

Brain sand or corpora arenacea of the pineal gland. In the pineal gland or epiphysis there are mineralized structures, the number of which increases with age – the so-called corpora arenacea (or "acervuli," or "brain sand"). These formations have a layered structure (hence the name – corpora arenacea) and festoon edges. Their sizes range from 5 microns to 2 mm and

resemble a mulberry berry in shape [16]. Chemical analysis of calcified cones of the pineal gland shows that they consist of compounds of calcium phosphate: hydroxyapatite, β-TCMF (beta-tricalcium-magnesium phosphate) [17]. There is also evidence of calcite in the "brain sand" [18].

The corpora arenacea of the epiphysis often serve as an anatomical reference point in the radiological examination of the head in patients after 30-40 years, since their number increases significantly with age [16]. The mechanisms of formation and function of "brain sand" are unknown. Some studies indicate that Alzheimer's patients have a significantly higher level of epiphysis calcification than other types of dementia [18]. It has also been found that with age, the pineal gland contains the same amount of fluoride as the teeth. In addition, there is a correlation of calcium and fluorine content in the epiphysis [17].

Otoliths. The inner ear contains minerals that are called otoliths or statocones and are part of the body of equilibrium. Otoliths have been found in all vertebrates and in parts of invertebrates, including many extinct creatures [19]. Statoconias in mammalian equilibrium organs are represented by elongated (10x1-3 μm) calcite crystals [20]. Otoliths can be products of cell secretory activity. Their displacement when changing the position of the body and the impact of acceleration causes mechanical irritation of the hair cells and the appearance of the corresponding nerve impulses [19].

1.3 Pathological biomineralization

The term "diseases of biomineralization" (calcification, pathological biomineralization, calcinosis, mineral dystrophy) includes such nosologies as deposition of calcium minerals in the walls of vessels and cardiac valves, cholera-, nephro-, sialo, prostatolithiasis, calcification of nodes in the thyroid gland, uterus, mineralization phenomena in almost all soft tissues of the body [5, 21, 22, 23, 24, 25, 26, 27]. The presence of biomineral

deposits that develop as a result of the development of a pathological process always has a significant impact on the development, course and consequence of a disease. In addition, this effect can be not only negative - in the form of complications of the disease with unfavorable prognosis, but also protective, that is, one that prevents dangerous or fatal complications or softens the course of the main nosology.

Depending on the localization in the human body biomineral formation has its morphogenetic and pathogenetic features. One of the most frequent localizations of pathological biomineralization is the cardiovascular system [25-27]. Other common nosologies include gallstone disease, nephrolithiasis, thyroid pathology, and prostatolithiasis. Often, biominerals are postponed in the lungs, salivary glands, oral and nasal cavities, much less likely to damage the organ of vision. The formation of calcifications in the tissue of tumors should be noted separately.

Cardiovascular calcification. In the cardiovascular system, PBM is most commonly found in complicated atherosclerosis (atherosclerotic plaques with calcification in the aorta, blood vessels, and cardiac valves), Menkenberg arteriosclerosis, phleboliths [28, 29]. Organo-mineral aggregates of calcium compounds formed on cardiac valves are called cardiolites in some literature [5, 8]. The greatest clinical and socio-economic importance is the PBM, which complicates the course of atherosclerosis. Cardiovascular calcification is considered an important predictor of morbidity and mortality in atherosclerotic vascular lesions [25, 29].

Recent works had raised the question that different forms of biomineralization in vessels have different biological properties and clinical behaviour [30]. Such PBM pattern as "microcalcification" is characterized by chronic inflammation in the pathological focus and the risk of atherosclerotic plaque rupture. On the other hand, the formation of large leaf-like

calcifications in the aorta, blood vessels, and heart valves corresponds to the pattern of PBM "macrocalcification", which in contrast, stabilizes the atherosclerotic plaque and serves as a barrier to inflammation [30].

Hydroxyapatite is the preferred mineral for cardiovascular PBM [29, 31].

Gallbladder. In the gallbladder (GB), mineral formation occurs not only in its cavity, but also in the walls - in cases of such nosologies as porcelain gallbladder or gallbladder cancer [32].

More common pathology is cholelithiasis, with the formation of stones in the organ cavity with organic and inorganic component. A favorable environment for the formation of stones is a violation of the composition of bile and a number of diseases of the GB and biliary tract [5]. The organic constituent of gallstones is represented by cholesterol, bilirubin and biliverdin (pigment stones). The inorganic part of the stones contained the following calcium-containing minerals: carbonates (faterite, aragonite, calcite, dolomite), phosphates (velocity). Pigments with high calcium content are particularly important - palmitate and calcium bilirubin, which are white and black in color [5].

The mineral component of the GB wall under conditions such pathology as porcelain gallbladder (PGB) was represented by hydroxyapatite [33]. Perhaps this is due to different conditions of mineral formation in the cavity and wall of the GB.

Kidney and bladder calculus stones. The basis for the formation of kidney stones and the urinary system is the violation of urinary homeostasis due to a number of diseases and the influence of external (mainly dietary) factors [34]. The formation of concrements is facilitated by an increase in the concentration of inorganic phosphorus (> 75 mmol / l), total calcium (> 7 mmol / l), magnesium (> 5 mmol / l), oxalic acid (> 0.25 mmol / l) and uric acid (> 4 mmol / l) in the urine. A

significant influence on the formation of minerals in the urinary system is fluctuations in pH. Thus, the daily average pH of a healthy person fluctuates within 5.5-6.5, under conditions of formation of oxalate stones the pH fluctuates within 4.8-7.0; in the conditions of urate - 4,5-5,8; under the conditions of phosphate - 6,0-7,7 [35]. The most common in the urinary system are oxalate stones (uevelite minerals, weddellite), phosphate stones (minerals struvite, hydroxyapatite, brucite, whitlockite) and urates (minerals uricite, uric acid dihydrate and ammonium urate) [35].

Pathological biomineralization of the thyroid gland. In the thyroid gland, calcifications occur in both benign and malignant tumor pathology [36]. Among all thyroid diseases, calcification is most commonly found in papillary thyroid cancer (PTC) [37, 38]. Based on the clinical features of pathologic biomineralization, Bai Y. et al. (2009), PTC calcifications were divided into psammoma bodies (PB), stromal calcification, and ectopic bone formation (ossification) [39]. PBs are characterized as spherical calcified cells, which are constructed of concentric plates and are an important diagnostic criterion for papillary thyroid cancer [36, 40, 41]. Ossification or bone formation was recorded in cases where the bone matrix and osteocytes were identified. All calcifications that did not fit the definition of PB and ossification were considered stromal calcification [39]. In addition to PTC, PBM is found in nodal (follicular and medullary cancer, nodular goiter, adenomas) and diffuse thyroid pathology (autoimmune thyroiditis, thyrotoxicosis) [36]. Typically, in these cases, the capsule of the nodes, the walls of the vessels, the stroma and the colloid of the follicles are subject to calcification. Hydroxyapatite mineral formation is characteristic for PBM of thyroid gland [37, 41, 42].

Prostatic lithiasis (prostatoliths). Ectopic mineral formation in the prostate gland can be realized due to the influence of such etiological factors as chronic inflammation,

congestive phenomena in the gland, urinary reflux from the urethra with intravesical obstruction, malformation of the prostate gland and seminal vesicles; specific inflammation, deficiency of protein-inhibitors of calcification. Prostatoliths are found in the alveolar glands and ducts of the prostate. According to a number of studies, including our own observations, hydroxyapatite is the predominant mineral among prostatoliths [43]. Some studies indicate that there are also oxalates among prostatoliths [44].

Sialolites and dentolites. The conception and formation of the salivary gland and dentolite stones are significantly influenced by saliva composition and parameters of saliva and inflammatory processes (sialoadenitis, caries). Sialolites and dentolites are characterized by similar morphology and chemical-phase composition. These biominerals have a concentric-layered structure: uneven layers of inorganic matter are separated by thin layers of organic matter [10]. The form of sialoliths is often determined by the structure of the salivary gland apparatus and its excretory ducts. With regard to the crystal-chemical composition of sialolites and dentolites, in addition to the predominant hydroxyapatite, there are also other phosphates of calcium - brushite, octacalphosphate and whitlockite [10, 11].

Pulmolites. Pulmonary alveolar microlithiasis (PAM) is a rare disease characterized by intra-alveolar deposits of calcium compounds as small round stones [46]. The etiology of the disease is still unknown, it is suggested that the cause is a hereditary defect of enzymes, since a large part of the PAM (about a third) has a family heredity [47]. PAM is characterized by the presence of many small nodules, calcified cells along the interparticle septa, bronchial-vascular bundles and pleural lesions in the form of fibrosis, subpleural cysts [46]. PBM in lung tissue often occurs as a result of tuberculosis (Ghon's

focus), chronic inflammation, and as a result of diffuse pulmonary calcification (DPC) [48]. DPC is observed in hyperparathyroidism, chronic renal failure, vitamin D intoxication, but has never occurred in the absence of other diseases. In other words, this disease meets the definition of metastatic lung calcification [48].

Ophthalmolites. PBM in the organ of vision is extremely rare. Among the ophthalmic pathology that is accompanied by mineralization can be called choroidal osteoma, idiopathic choroidal sclerosis, tophus-like scleral calcification, intraocular bone metaplasia and calcification in chronic inflammatory diseases [49]. The structure of biominerals and their phase composition was investigated in chronic inflammatory diseases. It was found that the main mineral component is hydroxyapatite with a defective lattice and small crystal sizes [49].

Rhinoliths. Mineralized objects in the nasal cavity are called rhinoliths [50]. They often form around foreign bodies or blood clots. In some cases, it is not possible to detect a foreign body in the nasal stone [51]. Occasionally rhinoliths are confused with osteomas of the nasal cavity, which are derived from the bones of the nasal sinuses.

According to the literature and our own observations, biomineralized nasal cavities are composed of calcium phosphate (hydroxyapatite), representing calcified detritus [52].

PBM in malignant tumors. Biominerals are closely related to secondary changes in tumor tissues, the presence of necrosis or necrobiosis foci. The large number of dead cells is a source of phosphates and calcium, which creates the conditions for the formation of minerals of the calcium phosphate group, mainly apatites [53]. Postpone of calcium deposits also occurs around the tumor node (in adjacent tissue), vascular walls, and lymph nodes [36, 53]. This type of PBM differs from classical dystrophic calcification. A specific type of tumor-associated PBM is PB, which is a characteristic diagnostic feature of some

tumors (PTC, papillary ovarian carcinoma, meningioma) [54]. The features of the occurrence, formation and biological behaviour of PB also distinguish them from the manifestations of dystrophic calcification [36, 55].

The behaviour of osteotropic metastases of various malignant tumors is also interesting. There are two types of metastatic bone lesions: osteolytic and osteoblastic metastases [56]. Osteolytic metastases are characterized by the dissolution of the mineral component of bone, which can lead to pathological fractures, osteoporosis, hypercalcemia. Conversely, osteoblastic metastases result in a tightening of the bone mineral tissue, which slows the spread of the tumor. Such tumors have a more favorable clinical course and prognosis [56]. Perhaps the main regulatory role in this process belongs to the cells of bone tissue - osteoblasts, whose function is enhanced under conditions of osteoblastic metastases.

An important component of physiological and pathological biomineralization is the organic-mineral aggregate. The interaction between the organic and mineral parts of the OMA is governed by certain laws. On the example of physiological biominerals, the mechanism of formation has been well studied, the regulatory function of the organic (protein) part of OMA (cells, activator molecules, and biomineralization inhibitors) has been established [57].

1.4 The concept of the "niche of biomineralization"

When studying the processes of biomineralization in soft tissues, there is a realization that the forming of biominerals is a complex open physicochemical system that interacts with the whole organism. This system consists of cells, non-cellular elements (stromal elements), molecular and metabolic nanoenvironment of biomineralization processes. The physicochemical environment of biomineralization includes elements of the circulatory system, the acidity of the medium,

the level of substrates (phosphorus, calcium, protons, carbon dioxide), hydrostatic pressure, the presence/absence of an appropriate matrix, oxygen level, enzymatic supply, regulatory (cytokine) supply or, maybe, elements of nerve regulation. The combination of all these elements forms a "biomineralization niche", in which there is a complex multicyclic process of formation of biominerals in the tissues of living organisms (Fig. 1.2).

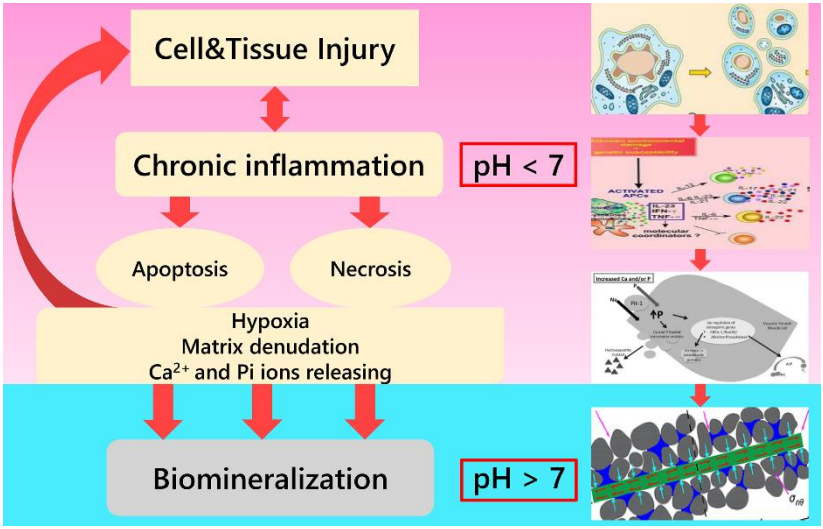

Figure 1.2 – Scheme of "niche biomineralization" in soft tissues

1.5 Cellular microenvironment of biominerals

In healthy people, the process of physiological mineral formation is limited by specialized tissues such as the skeleton and teeth. Throughout life, the formation of mineral tissue and calcium-phosphorus homeostasis are under the strict control of various regulatory systems of the body. For example, a change in the concentration of calcium ions in the serum even by 1 %

immediately leads to mechanisms that restore its equilibrium [5].

Cells - sources of mineralization in the vascular wall and soft tissues come from several precursors. These include resident half-stem cells, circulating mesenchymal cells from bone marrow stroma, subendothelial pericyte-like cells, vascular mesenchymal cells (also known as calcifying vascular cells), adventitious myofibroblasts, smooth myocytes (SMC - mature and immature), endothelial cells. The last two cells can be transformed into osteoblastic cells by epithelial-mesenchymal transformation [58].

Also known is intra-membrane ossification, which occurs in the case of direct mineralization of the matrix of mesenchymal cells that undergo osteogenic differentiation. However, endochondral ossification occurs in cartilage as an intermediate stage of mineralization [14]. For example, with the growth of long bone plates, chondrocytes take the place of future bone tissue, creating a gradient of chondrocytes at the stages of bone maturation: proliferation, pre-hypertrophy, hypertrophy, and apoptosis. Apoptotic bodies act as nucleation centres of calcium phosphate crystals produced by calcified cartilage, which acts as a matrix where microvessels grow, bringing endothelial cells and pericytes [59].

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endothelial cells and pericytes [59]. Chondroclasts, which derived from monocytes, dissolve calcified cartilage. The pericytes of the microcirculatory bed (MCB) under the influence of the calcified matrix and its components undergo osteogenic differentiation. Thus, osteogenic cells formed from pericytes become the source of bone matrix formation, osteons, matrix vesicles (MV), new crystals of hydroxyapatite that replace cartilage. Endotheliocytes and pericytes, upon maturation into osteoblasts or chondrocytes, begin to express "osteochondrogenic" genes and secrete MV in the extracellular matrix. The transformative effect of hydroxyapatite crystals on pericytes and vascular smooth muscle cell (VSMC), which in their presence acquire osteoblastic differentiation, was also found [60, 61].

Matrix vesicles. Initiation of calcification occurs inside the matrix vesicles (MV), extracellular entities originating from the cell membrane [62]. Calcified MV contain high levels of Ca^{2+}, which is bound to acidic phospholipids, phosphatidylserine and membrane phosphatases (including tissue nonspecific alkaline phosphatase (TNSALP). They hydrolyze various esters of phosphoric acid of natural origin, which eventually gives rise to the formation of calcium-phosphate minerals. The inner microenvironment surrounded by the membrane protects the nuclei of the mineralization in the pre-crystallization state before converting them to hydroxyapatite. With the rest, MV merge with the already existing crystals of hydroxyapatite, which leads to further mineralization [63].

There are several mechanisms for the formation of MV. MV was first described by Clark X Andersen in 1967 in the form of buds on degenerate cell membranes in bone matrix and bone cell cultures [64]. Subsequently, similar formations were found in other tissues, including blood vessels and cultures of endothelial cells, dendritic cells, and smooth myocytes [59].

They have been called different terms - extracellular vesicles, apoptotic bodies and microvesicles [65]. The composition of MB VSMC was thoroughly investigated and compared with MB of skeletal tissues. The molecular composition of MV of different origin, consisting of alkaline phosphatase, a considerable number of annexins and calcium minerals in the nucleation state, has been established [59, 65]. In the study of pathologic mineralization in atherosclerotic plaques, the possibility of MV formation by macrophages and possibly other inflammatory cells has recently been identified [15, 66].

1.6 Molecular nanoenvironment: pro- and anti-calcifying effects

Alkaline phosphatase and PHOSPHO1. TNSALP is a phosphatase that destroys a pyrophosphate mineralization inhibitor (PPi) [67, 68]. An additional phosphatase, PHOSPHO1, was recently detected in MV [69, 70]. In the absence of PHOSPHO1 deficiency, skeletal abnormalities, osteomalacia, and decreased TNSALP and PP levels appear in experimental mice [71]. In PHOSPHO1 deficient mice, TNSALP overexpression does not correct skeletal mineralization, regardless of correction of PPI level, since skeletal mineralization is completely absent in mutant PHOSPHO1 and TNSALP mice [70]. Therefore, it can be assumed that PHOSPHO1 is not a backup initiator of biomineralization. It has also been experimentally demonstrated in vitro that pharmacological inhibition of PHOSPHO1 in VSMC slows down matrix mineralization [69].

Ectonucleotide pyrophosphatase / phosphodiesterase (ENPP1). Ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) is a transmembrane glycoprotein that helps regulate bone and cartilage mineralization through the production of inorganic phosphate [10]. Based on the results of the study of animals with a double gene knockout of TNSALP and ENPP1,

it was found that these two enzymes are important for the balance of activity of other mineralization factors. Double knockout of the genes of these proteins corrects the deviations in biomineralization that occur in the event of simple damage (e. g., deletion) of the gene of each of them. ENPP1 functions as a maker of pyrophosphate mineralization inhibitors and under the influence of reducing the activity of alkaline phosphatase, a generator of extracellular inorganic phosphate (due to phosphodiesterase activity), which promotes the growth of crystals of minerals [71].

Gamma-carboxyglutamic acid matrix protein (MGP). A small MGP protein has been identified as a key inhibitor of vascular mineralization [72, 73]. It is known that in the absence or deficiency of this protein, mice develop rapid and clear vascular calcification [74]. It is believed that MGP can bind to bone-forming proteins (BMPs) and/or directly to calcium minerals. The rapid development of pathological biomineralization in the absence of MGP is explained by the non-alternative effect of BMP on the expression of genes responsible for osteoblastic cell differentiation. The formation of minerals is potentiated by endocytosis of calcium phosphate nanocrystals, which has an inductive influence on osteochondrogenic genes [75].

An interesting feature of MGP is its dependence on vitamin K, which is involved in post-translational modification by gamma-glutamyl carboxylation [76]. This is confirmed by the fact that warfarin, which blocks the activity of vitamin K, enhances ectopic biomineralization [77].

The matrix γ-carboxy glutaric acid protein is the first time obtained from the bone. To become fully functional, it requires vitamin K-dependent γ-carboxylation, but it is non-carboxylated MGP associated with vascular calcification. By binding to BMP-2, MGP blocks its activity against osteoblastic differentiation of VSMCs and differentiation of mesenchymal

cells. MGP also contacts to crystals of calcium salts and inhibits their growth [76]. Together with fetuin, they act as crucial regulators of the evolution of membrane-associated matrix vesicles [78].

Fetuin A (Heremans-Schmid α2 protein). Fetuin A is a calcium-binding protein that is produced mainly in the liver. Mice with an absent gene of fetuin A demonstrate the development of massive calcification of the lungs, blood vessels, and other tissues that somehow bypasses the aorta. Fetuin A globulin is a transporter of sex hormones in the blood that may be associated with climax and subsequent onset of progressive biomineralization. Whereas MGP, osteopontine (OPN), and osteoprotegerin (OPG) are local factors, fetuin A is a significant "systemic" circulating inhibitor of vascular calcification [79]. VSMCs are capable of incorporating serum fetuin A and depositing it in intracellular, membrane-bound matrix vesicles. They are released by cells into the matrix and become centres of crystallization of minerals there, but not when they are rich by fetuin A. Fetuin A, in combination with insoluble crystals, is called calcium-protein particles (CPP) in some studies [79-80]. The incorporation of fetuin A into the MMC is enhanced by extracellular calcium but not by extracellular phosphates. The entry of this protein into smooth myocytes (SMC) increases Ca^{2+} flow in them and is mediated by the activity of calcium channel annexin, which facilitates the inhibitory role of fetuin A on the mineralization of SMC [79]. Mice that do not produce fetuin A develop microcalcifications in small vessels and increase the deposition of calcium compounds in soft tissues [66].

Receptors of nuclear activator of nuclear factor kappa-B ligand (RANKL) and OPG. RANKL is a key factor in the resorptive osteoclastic activity that is triggered by lipid peroxidation products (LPO). OPG is a receptor-bait for RANKL, otherwise known as osteoclastogenesis inhibitory factor (OCIF). OPG belongs to the tumor necrosis factor (TNF)

family and is a cytokine receptor [81]. Activation of RANKL in vascular MMCs of mice occurs through the osteogenic differentiation factor Runx2 in a dose-dependent manner: initially, the products of LPO and reactive oxygen species (ROS) cause activation of Runx2, in response to which RANKL begins to be synthesized [82]. Earlier, it was found that mice deficient in RANKL and OPG receptors developed clear vascular calcification [83]. In other work, OPG has been shown to reduce vascular calcification in hypercholesterolemic mice [84]. The anti-mineralization effect of OPG is realized through the downregulation of the Notch1-RBP-Jkappa metabolic pathway and the subsequent reduction of Msx2 and TNSALP mediators [85]. OPG inhibits osteoclast differentiation and is a key modulator of bone resorption due to its function as a trap receptor for the activator of the nuclear factor κB receptor ligand (RANKL). It has been investigated that it prevents calcification of the media, possibly due to the immunomodulatory effect on the low-grade inflammatory process in the vessel wall [86].

In the study of OPG - / - knockout mice, they revealed active bone resorption due to activation of osteoclastic differentiation and a massive "leakage" of the bone tissue mineral component [87].

Fibroblast growth factor 23 (FGF23), Klotho, and chronic renal failure (CRF). FGF23 is synthesized in skeletal osteocytes in response to hyperphosphatemia on the basis of negative feedback [65]. This protein has a molecular weight of 30 kDa. FGF controls renal phosphate excretion by regulating renal sodium, a dependent phosphate co-transporter (NaPi2a and NaPi2c). Since hyperphosphatemia is a permanent metabolic consequence of CRF, renal proteins are involved in the regulation of calcium-phosphorus metabolism, namely, clotho, a protein that is expressed mainly in the distal tubules of the nephron [88]. It can act as a circulating form, or as a hormone. Klotho binds to FGF-23 receptors and opens to different cells

the ability to respond to FGF-23 by acting as a cofactor. FGF-23 and Klotho play an important role in the systemic regulation of phosphate homeostasis, reducing their resorption and decreasing vitamin D. After vitamin D levels, FGF-23 levels increase, reducing renal calcitriol synthesis due to the effect on hydroxylase 1α gene. FGF-23 also affects parathyroid hormone (PTH), being a negative regulator of its expression. On the contrary, PTH stimulates the secretion of FGF-23 from bone tissue. The Klotho-FGF-23 axis is assumed to function as follows: with the progression of CRF by the help of FGF-23, the activity of PTH and 1α - hydroxylase is suppressed, thus reducing the level of calcitriol, thus reducing the phosphate content [89]. In mice with "knockout" genes FGF-23 and Klotho developed a clear calcification of blood vessels and soft tissues. Experimental studies have shown that elevation of FGF-23 and Klotho protein deficiency can directly induce vascular calcification. It has also been established that Klotho can act as a circulating inhibitor of vascular calcification [88]. The overexpression of Klotho reduces the vulnerability of mice to CRF-induced vascular calcification, and conversely, in the absence of this protein, the susceptibility of blood vessels to pathogenic biomineralization caused by CRF is significantly increased [90].

Pyrophosphates (PPI). Pyrophosphates inhibit the formation of hydroxyapatite crystals. They are formed from nucleotides - nucleotide triphosphates - pyrophosphatase, which deficiency causes marked calcification of the copper, as well as the deficiency of the pyrophosphate transporter - ANK. Moreover, the mechanism of pyrophosphate-dependent accumulation of calcium in the vascular wall also encompasses the inhibition of osteochondrogenic transdifferentiation - the same process stimulated by phosphates [91]. Clarifying the mechanism of this regulation may pave the way for the creation of new pharmacological agents based on bisphosphonates.

Receptors advanced glycation end products (RAGE) and calgranulins. RAGEs play an important role in the pathogenesis of atherosclerosis and the development of concomitant pathological mineralization. It has been noted that their expression co-localizes with inflammation foci in unstable atherosclerotic plaques with microcalcification [92]. In addition, compatible localization and increased expression of RAGE with vascular SMCs that are subject to osteochondrogenic differentiation have been identified [93].

RAGE ligands quench soluble forms of these receptors. Serum levels of RAGE trap-receptors are inversely associated with vascular calcification, confirming the role of these receptors in pathological biomineralization. One of these ligands is the S100 family proteins: calganulins A, B and C (S100A8, S100A9, S100A12) [94]. It has been found that overexpression of the RAGE S100A12 ligand in vascular MMCs of mice with hyperlipidemia significantly enhances vascular mineralization, partially mediated through the activation of LPO [95].

Galectin-3 (Gal-3). An important receptor for LPO and advanced glycosylation end products is galectin-3. However, the role of galectin-3 in the development of atherosclerotic process is ambiguous. On the one hand, RAGE stimulation (including galectin-3) promotes the development of atherosclerosis through their proinflammatory effects [96]. But at the same time, galectin-3 contributes to the destruction of LPO products and glycosylation and their internalization (endocytosis), which has an anti-inflammatory effect [97]. In galectin-3-deficient mice, atherogenesis is increased on a diet rich in fat [30]. Galectin-3 and RAGE have been found to be important in bone formation and resorption, modulating skeletal tissue homeostasis [98]. Current studies on the role of galectin-3 and RAGE show different mechanisms of their action on pathological processes of biomineralization. To realize the effects of galectin-3, complete acquisition of the osteoblastic phenotype by vascular

vascular SMCs is required, causing them to activate β-catenin at the level of the nucleus. Runx2 lowers galectin-3, suppressing increased osteoclastogenesis, a key factor in osteogenic differentiation [2].

In addition, RAGE and galectin-3 play a crucial role in plaque ulcer and clinical sequelae due to vascular calcification and inflammation. In atherosclerotic plaque, RAGE provokes and exacerbates inflammation, which leads to the deposition of microcalcifications and the occurrence of plaque instability. Galectin-3, on the contrary, exhibits anti-inflammatory activity only after the calcification process has started, promoting the progression of deposition of calcium compounds and causing the formation of leaf-like macrocalcification in the vessel walls [94]. Despite the opposite effect on inflammation and different signaling pathways, RAGE and galectin-3 enhance vascular mineralization.

Due to the formation of macro- (galectin-3) and microcalcification (RAGE), the activity of these proteins, respectively, leads to the stability or instability of the atherosclerotic plaque. General information about the role of molecular microenvironment in the development of biomineralization is summarized in table 1.1.

Table 1.1 – Pro- and anti-mineralization factors in the development of biomineral deposits

Factor	Transcription	Action
Tissue nonspecific alkaline phosphatase	TNSALP	Promotes mineralization
Phospho1 phosphatase	PHOSPHO1	Promotes mineralization, it is a part of the content of MV
Ectonucleotide pyrophosphatase / phosphodiesterase	ENPP1	Produces inorganic phosphate, promotes mineralization
Fibroblast Growth Factor 23, protein Klotho	FGF23, Klotho	Counteract hyperphosphatemia and mineralization
Matrix Gla-protein	MGP	Suppresses mineralization
Osteoprotegerin	OPG	Suppresses osteoblast differentiation, a modulator of bone resorption
Receptors of nuclear activator of nuclear factor kappa-B ligand	RANKL	Promotes osteoclastogenesis, a promoterization factor
Fetuin A	Fetuin A	Calcification inhibitor
Pyrophosphate	PPi	Suppresses the development of biomineralization
Galectin-3	Gal-3	Inhibits increased osteoclastogenesis, promotes macrocalcification
Receptors advanced glycation end products	RAGE	Proinflammatory factor, promotes microcalcification, plaque instability
Calgranulins A, B, C	S100A8, S100A9, S100A12	They sequester calcium, partly contributing to calcification

1.7 Metabolic features of biomineralization

The metabolic atmosphere or microenvironment of the foci of biomineralization is an important component of *the niche of biomineralization*. It is produced by the structural and molecular components of the biomineralization niche, creating a specific physico-chemical microenvironment. The metabolic microenvironment includes such indicators as the level of carbon dioxide and oxygen saturation, acidity, tissue fluid pressure, calcium and phosphorus ions content, perfusion, and the level of apoptotic processes.

It is the metabolic component that determines the formation of certain crystalline phases and determines the directions of biomineralization processes under different organ locations. For example, the high level of bicarbonate ions in the pancreas and gallbladder causes the formation of biominerals in the form of calcite and its related phases (vateritis, dolomite).

Features of biomineralization in atherosclerosis. For the normal performance of its functions, the vascular wall requires such properties as elasticity and elongation. Therefore, solid, rigid deposits of calcium compounds in the arterial walls have important implications for hemocirculation. Obviously, increased rigidity of the vessel wall impairs vasomotor activity. Pathological biomineralization of blood vessels is widespread, affecting approximately 60 % of people over 60 and 80 % of people over 80 [65].

It is a well-known fact that vascular calcification is an unfavorable sign of the development of atherosclerosis and is a predictor of cardiovascular pathology and mortality of patients [99]. The plaque rupture is a serious destructive consequence of atherosclerosis [65]. Biomechanical analysis shows that rigid (dense) inclusions - calcifications in the wall of the arteries increase the pressure on the stressed surface of the vessel in the areas of direct influence of the negative factor, thus increasing the risk of rupture [99]. Further studies of this problem show that

the likelihood of such a complication increases significantly in the case of close localization of two microcalcification deposits [25]. In the case of localization in the heart valves, the consequences may be more severe as there is a violation of the hemodynamic flow. For example, in the case of calcified aortic stenosis, backflow from the left ventricle is blocked. In general, the biomineralization of blood vessels and heart valves leads to such adverse clinical effects as systolic hypertension, left ventricular hypertrophy, coronary ischemia, congestive heart failure, atherosclerotic plaque rupture, thrombosis, myocardial infarction.

The cardiovascular system is characterized by the formation of biomineral compounds of calcium phosphate salts in different ratios between Ca and R. The general information on this subject is summarized in table 1.2.

Apoptosis and biomineralization in soft tissues. In the foci of chronic inflammation, cellular damage and death, including DNA damage, autophagy, and apoptosis, are widespread. In the process of apoptosis, vascular SMCs release MV and apoptotic bodies capable of concentrating calcium and forming calcium phosphate crystals [100]. Thus, factors contributing to cell death also enhance biomineralization processes. For example, prelamin A blocks DNA repair and promotes osteoblastic differentiation and mineralization of vascular cells [101].

Table 1.2 – The main compounds of calcium phosphate, which involved in the processes of pathological biomineralization of the cardiovascular system

Name	**JCPDS Formula and Number (Joint Committee on Powder Diffraction Standards)**	**Ca / P ratio, at %**	**Density, g / cm-3**
Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)_2\ H_2O$ 9-0347	0.5	2.23
Monocalcium phosphate anhydrous (MCPA)	$Ca(H_2PO_4)_2$ 9-0390	0.5	2.58
Dicalcium phosphate dihydrate (mineral - brushite)* (DCPD)	$CaHPO_4\ 2H_2O$ 9-0077	1.0	2.32
Dicalcium phosphate anhydrous (mineral - monetite)* (DCPA)	$CaHPO_4$ 9-0080	1.0	2.89
Octacalcium phosphate (OCP)	$Ca_8H_2(PO_4)_6\ 5H_2O$ 26-1056	1.33	2.61
β-tricalcium phosphate (β-TCP, Mg β-TCMP)	β-Ca_3PO_4, β-$(Ca,Mg)_3PO_4$ 9-0169	1.5	308
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$ 76-0694 або 9-0432	1.67	3.16

* Natural minerals of brushite and monetite do not fully correspond to the idealized DCPD and DCPA orthophosphates, respectively

2 DISEASES ASSOCIATED WITH BIOMINERALIZATION: PATHOMORPHOLOGICAL FEATURES

Diseases associated with biomineralization can be defined as diseases in where the formation of biomineral deposits, which have a significant impact on their course and prognosis of a particular pathology. Today, many common human diseases are known, which are associated with the formation of biominerals of a predominantly calcium-phosphate nature [102]. Among these nosologies, atherocalcinosis of vessels and heart valves, chole-, nephro-, sialo-, pancreatic-, prostatolithiasis, calcification in cancer of the thyroid gland, uterus and other localizations are the most common [5].

An epidemiological study was conducted on diseases associated with biomineralization, which contained samples of PBM studied in this work and are most common in the Ukrainian population: coronary heart disease (CHD), cholelithiasis (ChL), prostate cancer (PC) and thyroid cancer (TC).

The causes that lead to an increase in the prevalence of diseases associated with biomineralization are extremely diverse and diverse: environmental, socio-economic, climatic, metabolic disorders.

2.1 Pathological biomineralization in the cardiovascular system

The socio-economic significance of CHD is confirmed by the fact that it accounts for 2/3 of deaths from cardiovascular disease [103]. The presence of biomineralization in the cardiovascular system is an important predictor of morbidity and mortality. Thus, the deposition of calcium deposits in soft cardiovascular tissues disturbs the biomechanical functions of these tissues and leads to complications such as heart damage, myocardial infarction, and stroke [104], so their study is of great interest.

Comparison of the prevalence of coronary heart disease in the population of Sumy region and Ukraine shows a predominance of indicators of this pathology at the national level during the period 2012-2016 (Fig. 2.1). But the average annual growth rate of CHD in the Sumy region was 101.86 % as opposed to the Ukrainian average - 95.9 %. The average prevalence of coronary heart disease each year has increased by 1.86 % in the Sumy region, while the average Ukrainian index is negative (-4.10 %).

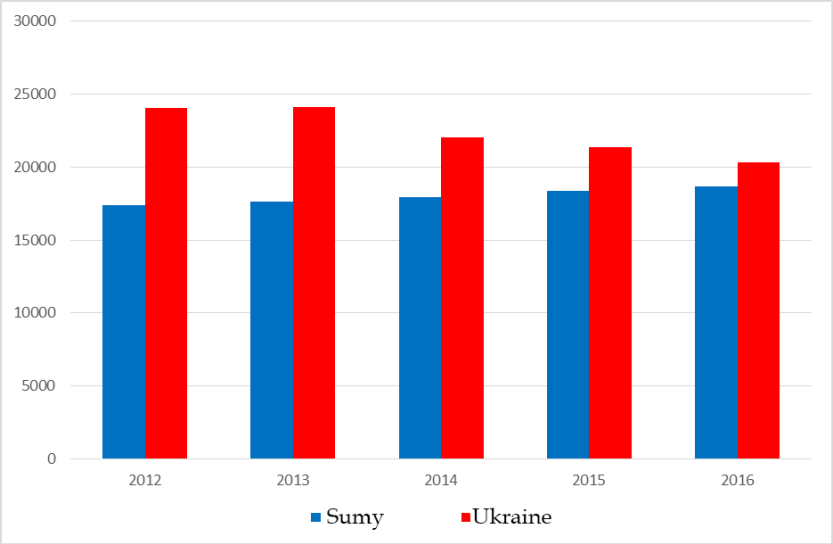

Figure 2.1 – Comparison of CHD prevalence among the population of Sumy region and Ukraine during 2012-2016

During the observation period it was found that the incidence of coronary heart disease in the Sumy region is lower than the average in Ukraine (Fig. 2.2). At the same time, the following trends are noted: the incidence of coronary heart disease in Ukraine is decreasing, and in the Sumy region its slow growth is observed. On average, every year the incidence of coronary heart disease has increased by 0.02 % in the Sumy

region, while the average Ukrainian indicator was negative (-8.45 %).

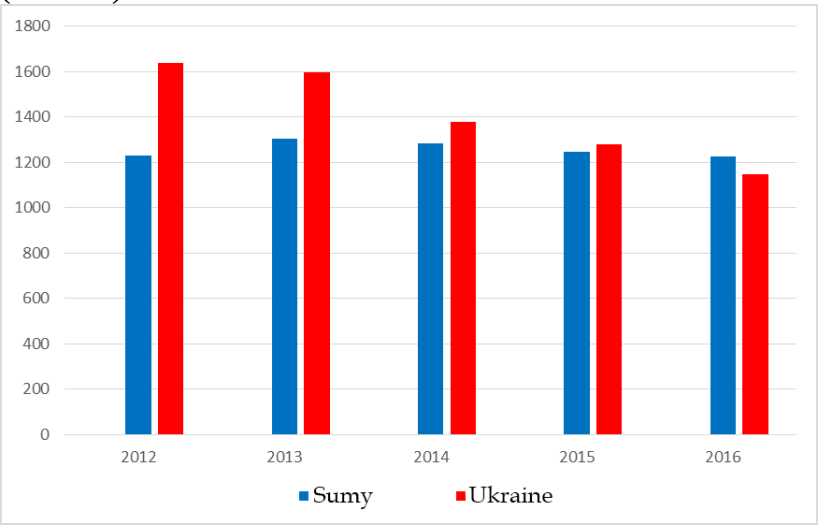

Figure 2.2 – Comparison of CHD incidence among the population of Sumy region and Ukraine during 2012-2016

Structural changes in the demographic situation in the countries of Europe and Ukraine towards the increasing population of older age groups leads to an increase in degenerative-metabolic diseases. In this regard, the prevalence of aortic atherosclerotic lesions and valvular structures of the heart is increased, which significantly impairs the quality of life and prognosis for patients [105]. For example, the prevalence of heart valve lesions of different origins is 20-30 % of patients over the age of 65 [106] and reaches 48-57 % in eighty-year-old patients [2, 107, 108].

The biomechanical properties of histological tissues depend on the microarchitecture of the extracellular matrix (ECM) [109]. Calcification disrupts the soft tissue histoarchitectonics by depositing solid mineral deposits [110]. Loss of biomechanical stability of the cardiovascular system

leads to acute or chronic adverse complications, such as violation of the integrity and deformation of the walls of the vessels and heart, valve apparatus, heart attacks. Two types of cardiovascular biomineralization are of paramount importance for human health: micro- and macro-calcification [2].

It is widely accepted that microcalcification, or so-called spotty calcification, associated with increased cardiovascular mortality [111]. The biomechanical aspects of microcalcification are discussed in several recent works, which discuss the pressure distribution in the atherosclerotic plaque and the mechanism of its rupture by mineral deposit [92]. Diffuse calcifications, which form solid fragments of more than 5 mm, usually located in the form of sheets (sheet-like calcification), are defined as a manifestation of macrocalcification [2]. In contrast to spot calcification, macrocalcification stabilizes the atherosclerotic plaque and serves as a barrier to inflammation [111].

Damage to the aortic valve (AV) with calcification of the valve apparatus is the most common heart valve disease in Europe and the United States, especially in older people [110]. Thus, in developed countries, about 50 000 operations of cardiac valve replacement are performed every year due to this pathology [112].

This has led to an increase in the number of elderly patients with non-rheumatic valve involvement: they are more likely to have ischemic heart disease, congestive heart failure, undergo surgery for myocardial revascularization, type II diabetes, and chronic kidney disease [109]. According to the Euro-Heart Survey on Vascular Heart Disease, degenerative etiology dominates for aortic stenosis (81.9 %), predominates for mitral (61.3 %) and aortic (50.3 %) insufficiency. Only for mitral stenosis, rheumatological factors remain the main etiological factors (85.4 %) [109]. The most common pathology of acquired heart defects is non-rheumatic aortic stenosis, which

is also called calcified aortic stenosis (CAS), since in its pathogenesis the main role belongs to the process of biomineralization of the shutters and valve rings [111]. In addition, the mean age of patients with CAS is higher than that of other valve pathology. Thus, the demographic-induced increase in degenerative-metabolic diseases of the human body leads to an increase in the proportion of calcification of the heart valves.

The degree of mineralization of the aortic valve is estimated by echocardiography:

I degree of calcification corresponds to the focal deposits of calcium compounds in the cusps and commissures;

II degree – rough calcification of cusps and AV commissures, which does not cover the areas of attachment of cusps;

III degree is the massive deposition of calcifications with the transition to the fibrous valve ring, the aorta and the outlet of the left ventricle, the anterior cusp of the mitral valve [113].

As you can see, in the case of severe atherosclerotic lesions, the combination of different "lodges" of atherosclerosis is combined into one pathological focus.

According to the classical definition of WHO, the concept of atherosclerosis includes "a variable combination of damage to the inner lining of the arteries, which consists of deposits of lipids, hydrocarbons, blood components, connective tissue and minerals" [92, 103]. The aorta is the most common localization of the atherosclerotic process. The general mechanism that leads to the development of biomineralization in blood vessels is being actively researched but is not yet completely understood. An atherosclerotic lesion with the presence of calcifications in classical pathology is considered a manifestation of complicated disease. Still, microcalcifications begin to emerge at the lipid stage [99].

In the developed countries of the European Community and North America, the modified Virmani R. (2003) morphological classification is used, which is a modification of the AHA (American Heart Association) classification [114]. All morphological changes in the walls of the vessels are divided into groups of early changes, unstable and stable disorders. Early changes include adaptive intimal thickening (AIT), intimal xanthoma (IX), pathological intimal thickening (PIT), and early fiber atheroma (EFA). Unstable (prevulnerable) conditions are considered late fibroatheroma (LFA), thin fiberoatheroma (TCFA), ruptured plaque (PR) and complete rupture of plaque (HR). A stabilized atherosclerotic condition is defined as fibrous calcified plaque (FCP). Some authors believe that the first calcium deposits in atherosclerotic lesions appear at the stage of late fiberoatheroma [92].

Obviously, the deposition of calcium phosphate minerals in the aortic wall, affected by the atherosclerotic process, takes some time from the moment of the onset of the underlying disease. In our study, this is confirmed by comparing the average age of patients in the aortic control group and the aortic group with biomineralization available, corresponding to 68.43 ± 1.32 (biomineral) and 51.8 ± 2.56 (control) ($p < 0.05$) years respectively [115].

Macroscopic examination of the aortic tissue with biomineralization revealed that, against the background of atherosclerotic lesions, calcium compounds were deposited in the area of the inner lining of the vessel. In cases of expressive stages of development of the pathological process, calcification also extended to the middle sheath. Histological examination showed a typical list of pathological changes in the aortic wall: focal deposition of lipids with the formation of cholesterol cysts, sclerotic changes with thickening of collagen and elastic fibers, edema, deposition of calcium deposits of different morphology and size, chronic inflammatory infiltration.

An element such as inflammation can occur as a reaction to atheromatous detritus and biomineralization. The significance of chronic inflammation in the development of pathological biomineralization in recent years has been given an extremely important place. It is believed that the predominance of pro-inflammatory stimuli in the development of atherosclerotic process leads to the development of prognostically unfavorable microcalcification, and in the case of the predominance of anti-inflammatory stimuli occurs macrocalcification, which contributes to the stabilization of atherosclerotic plaque [111].

A standardized set of immunohistochemical markers: MPO, CD68, S100A8, S100A9, Caspase3 and OPN were used for the immunohistochemical study of pathological biomineralization processes under different localizations in the soft tissues of the human body. The results of the immunohistochemical study were evaluated by counting stained cells in a field of view with a diameter of 1 000 μm in the environment of the morphometric programs "Pannoramic Viewer 1.15.4" and "SEO Scan Lab 2.0".

One of the central cores of our work was the study and clarification of the role of calcium-binding proteins of the calgranulins A (S100A8) and B (S100A9) and the calprotectin complex S100A8 / S100A9 in pathological biomineralization processes (Fig. 5). Calgranulins A and B are produced and secreted by a wide range of cells, but most of them contain neutrophils (up to 40 % of cytoplasmic proteins) and cells of the macrophage row (up to 10-15 % of cytoplasmic proteins) [116]. Therefore, the neutrophil markers of myeloperoxidase (MPO) and the CD68 macrophage differentiation cluster were used to identify the major "suppliers" of S100A8, S100A9 proteins and their heterodimeric complex S100A8 / A9. CD68 expression also occurs in some endothelial cells and fibroblasts, but they can be easily distinguished by specific cell morphology and localization.

Assessment of the level of elimination of cells in the pathological cell was performed using a marker of apoptosis caspase 3 (Casp3). The advantage of using Casp3 is that this marker directly indicates irreversible apoptotic cell changes, which takes precedence over other markers (eg, bax).

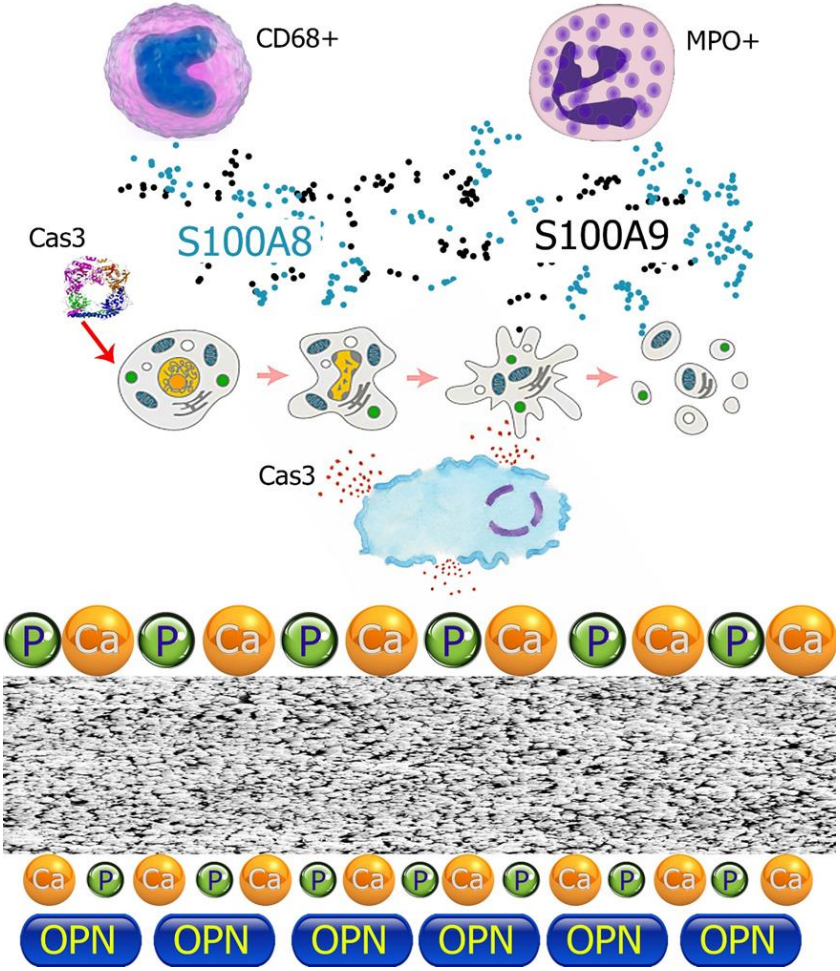

Figure 2.3 – Scheme of the role of calgranulins A and B in the development of pathological biomineralization of soft tissues

The death of a large number of cells leads to the creation of conditions for the development of biomineralization processes: calcium ions and phosphate groups from nucleic acids and ATP are released from the calcium depots of the dead cells, and a connective tissue matrix can be used as a matrix for deposition of calcium biomineral deposits. In some sample groups (aorta, aortic valve), monoclonal antibodies against type I collagen were used to detect it, since this protein is a natural matrix for bone mineralization. Immunohistochemical determination of OPN protein was used to evaluate the interaction of soft tissues and biominerals. This protein is known to be multifunctional with cytokine activity, has proinflammatory properties, and is secreted by a number of inflammatory infiltrate cells and soft tissues in response to the occurrence of calcium phosphate biominerals [117]. It is also known that it is able to interact with hydroxyapatite, inhibiting the growth of its crystallized forms [118].

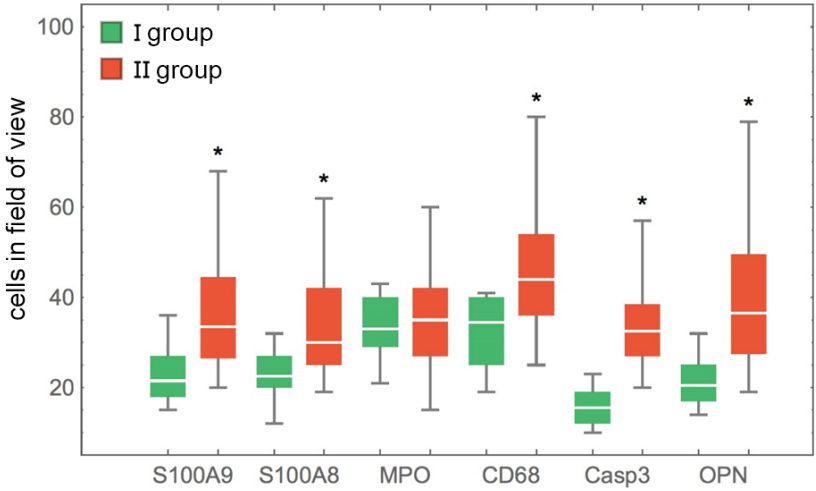

Figure 2.4 – Results of immunohistochemical study of aortic tissue. The average number of immuno-positive cells in the field of view. * - statistically significant values ($p < 0.05$)

Therefore, in comparing the results of immunohistochemical study of protein expression in aortic tissue I and II groups, a significant difference was found between the results of groups for markers of $CD68^+$ cells of the macrophage row ($p < 0.001$), components of the calprotectin complex - calgranulin A (S100A8) – $p < 0.01$) and B S100A9 - ($p < 0.001$), the Casp3 apoptosis marker ($p < 0.05$) and the OPN biomineralization marker ($p < 0.001$). For the MPO neutrophil marker, no significant difference between the parameters of the studied groups was detected ($p > 0.05$) (Fig. 2.4).

The difference between the results of the immunohistochemical study of the expression of CD68, S100A8, S100A9, Casp3 and OPN in the 1st and 2nd groups of aortic specimens indicates their role in the processes of biomineral deposits formation under the conditions of the atherosclerotic process. Obviously, the "calgranulin" pressure on the cells of the aortic wall and especially its inner layer, is carried out by cells of the macrophage row, which are high in saturation and can be noticed without any special studies already at the stage of analysis of microslides, stained with hematoxylin. The amount of calgranulin B was estimated to be higher, but the level of calgranulin A was also higher in mineralized tissues. The formation of the heterodimer calprotectin complex is confirmed by immunofluorescence study with the simultaneous use of two markers using Alexa 488 and Alexa 555 tags (Fig. 2.5).

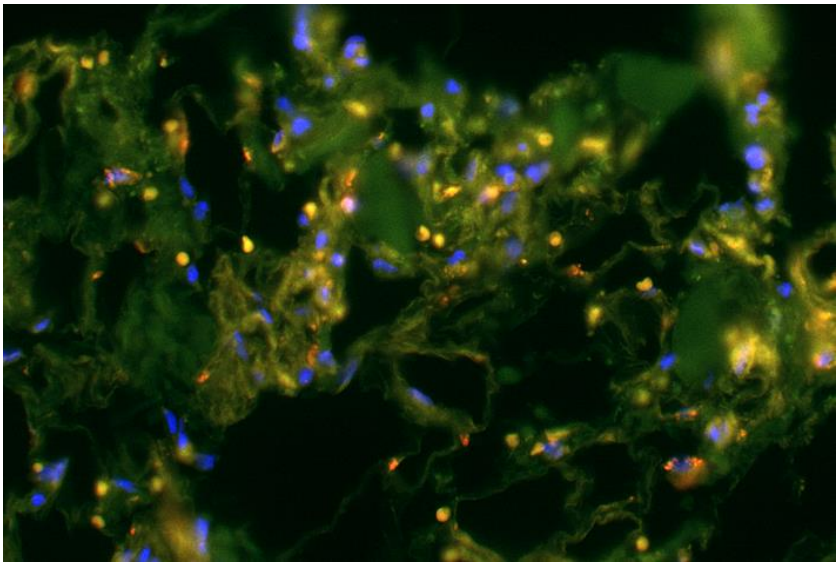

Figure 2.5 – Immunofluorescence study of S100A8 and S100A9 expression in group I aortic tissue co-localized as calprotectin. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x200

In the study of the presence of calprotectin in the tissues of the aorta using immunofluorescence antibodies, it is noticeable that in mineralized samples significantly more calprotectin complexes (in the photo is shown in orange) are formed (Fig. 2.5, 2.6).

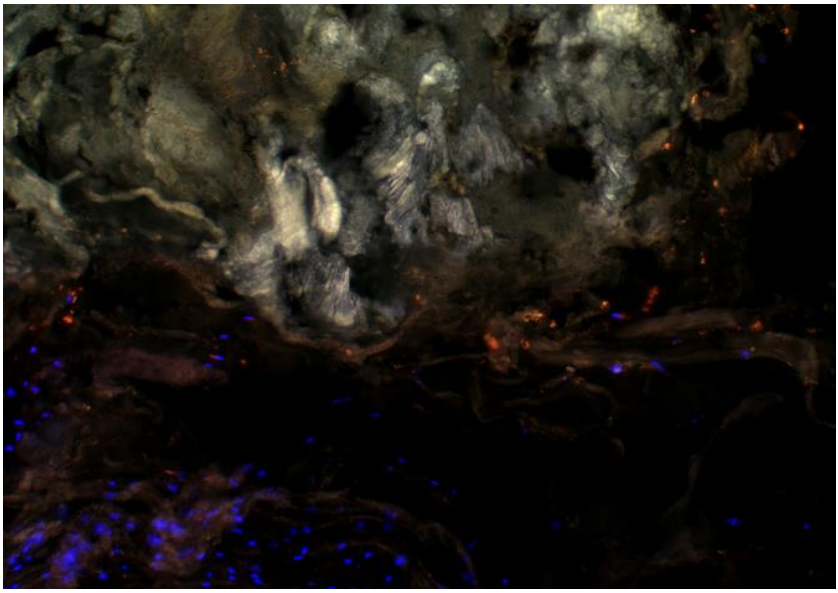

Figure 2.6 – Immunofluorescence study of S100A8 and S100A9 expression in group II aortic tissue co-localized as calprotectin at the border of the atheromatous plaque. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x100

Immunohistochemical study of the mineralized aortas of group I revealed high expression of the proapoptotic marker Casp3 in the areas of atherosclerotic plaque development, around small vessels and biomineral deposits, which was consistent with the data obtained on calprotectin activity. The expected result was the detection of OPN protein around atherosclerotic plaques and biomineral deposits, which were accompanied by inflammatory infiltration.

The results obtained using applied materials science methods for the study of group I aortic specimens clearly indicate the predominance of the structural defective carbonate-

containing apatite B-type among the biominerals (CO_3^{2-} ions replace PO_4^{3-}). These results were obtained by SEM with microanalysis, IR spectroscopy, TEM with ED, and X-ray spectroscopy.

The close interaction of the mineral and organic components of the wall of the atherosclerotic aorta leads to the formation of a structure by type of biocomposite material [119]. We found an interesting difference in the morphology of crystals of micro- and macrocalcifications (Fig. 2.7). Microcalcifications had sharp edges, radiant or irregular in shape, which may contribute to the inflammation and destruction of the soft tissue of the atherosclerotic plaque and its rupture. On the other hand, the macrocalcifications had a regular shape with relatively smooth edges and uniform thickness, possibly due to their formation on the smooth surface of the biological tissue (for example, connective tissue coating of atherosclerotic plaque). This process took place by a matrix-directed growth mechanism on an organic substrate under favorable conditions in the absence of inflammation and other stress effects.

Figure 2.7 – Scheme of placement of micro- and macrocalcifications in the walls of the aorta

The origin and development of pathological biomineralization in the heart valves affected by the

atherosclerotic process requires some time from the onset of the underlying disease. In our study, this is confirmed by the mean age of the patients, which corresponds to 68.91 ± 1.49 years.

Macroscopic examination of the aortic heart valves showed that at atherosclerotic lesions, biomineral deposits were localized in the cusps or in the fibrous ring. Histological examination revealed a typical list of pathological changes in the tissues of the heart valves: thickening of the fibrous layer and elastic fibers, focal deposition of lipids, myxomatous changes, phenomena of edema, deposition of calcium deposits of various morphologies and sizes, chronic inflammatory infiltration. Similar pathohistological signs accompanied the atherosclerotic process. They are often associated with secondary inflammation in response to atheromatous detritus and pathological biomineralization. In the latter case, a typical "circulus vitious" was formed: under the influence of pro-inflammatory stimuli, deposits of calcium in the form of grains, splinters, or granules were deposited, which irritate the surrounding tissues, causing inflammation [2, 94].

Pathological biomineralization is commonly thought to begin with the aortic valve and then extend to the mitral valve, left ventricle, and interventricular septum. Biomineralization dramatically alters the elasticity and elongation of the tissue of the valve apparatus, contributing to the development of its insufficiency, left ventricular hypertrophy and reduced cardiac muscle contractility in the future [65].

It is known that cardiovascular calcification is an unfavorable sign of the development of atherosclerosis and is a predictor of cardiovascular pathology and mortality of patients [99]. Particularly dangerous may be the destruction of an atherosclerotic plaque located on the valve petals or in the area of communication with the muscles [65]. There is an interesting opinion that microcalcifications have a higher damaging potential for tissue rupture [99]. A particularly dangerous

situation arises in the case of close localization of two microcalcification deposits [25]. Conversely, the formation of leaf-like calcifications in the cardiovascular system ("macrocalcification") stabilizes the atherosclerotic plaque and may act as a barrier to inflammation [2].

Current studies show the important role of Toll-like receptors 2 (TLR2) and TLR4 in the development of calcified aortal valve (CAV). Thus, expression of TLR2 and TLR4 causes osteogenic phenotypic changes in AV interstitial cells [121]. Involvement of (TLR2) and TLR4 in biomineralization processes draws attention to the agonist of these receptors, the calprotectin complex [S100A8 / S100A9], consisting of calgranulin A (S100A8) and calgranulin B (S100A9) [122].

In recent years, osteopontin, which is a proinflammatory multifunctional cytokine and a promising marker of the presence and degree of AV mineralization, plays an important role [123].

The deposition of calcium compounds in CAV occurred both in the form of coarse deposits and in the form of fine grains, crumbs, which encrusted connective tissue fibers. The affected valves were characterized by sclerosis and tissue hyalinosis, focal lipid deposition, edema, myxomatous changes, thickening of elastic and collagen fibers. This was especially the case for type I collagen, which has the highest tendency for calcification [124]. Important pathohistological changes in CAV were inflammatory mixed-cell infiltration by cells of the macrophage origin (histiocytes, macrophages - CD68-positive cells and neutrophils (MPO-positive cells).

An immunohistochemical study of the expression of calgranulin A (S100A8) in CAV tissue showed focal expression in inflammatory infiltrate cells, but no significant extracellular expression was detected. A study of the distribution of calgranulin B (S100A9) showed its pronounced expression in the tissues of calcified aortic valves both extracellularly and intracellularly in most resident valve cells and circulating

inflammatory cells. In the IHC - the study of CAV tissue with fluorescent antibodies to S100A8 (Alexa 488, red) and S100A9 (Alexa 555, yellow), the co-localization of these molecules in the calprotectin complex stained the cytoplasm of S100A8 / S100A9 cells in orange (Fig. 2.8).

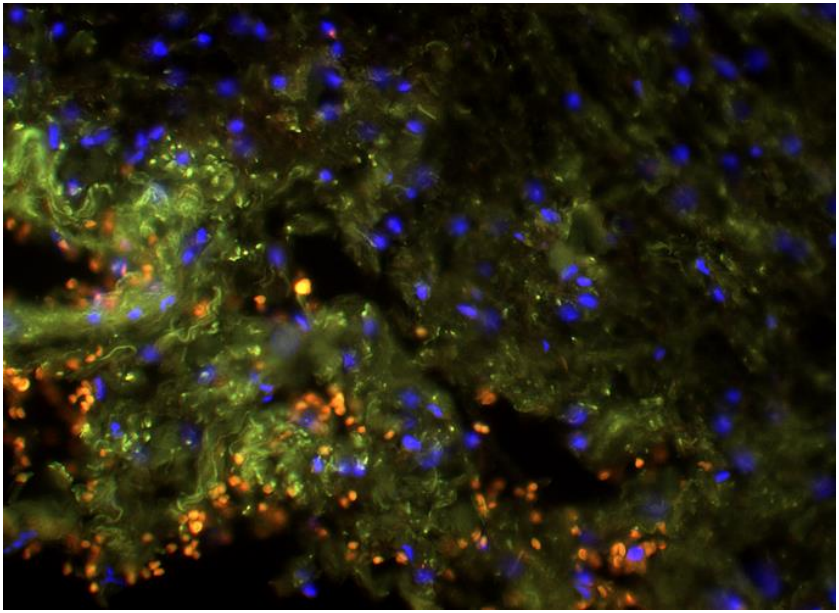

Figure 2.8 – Immunofluorescence study of S100A8 and S100A9 protein expression in tissue of group I aortic valves co-localized as calprotectin. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x200

A study of apoptotic activity in CAV tissues, conducted by studying the level of expression of the marker of apoptosis Casp 3, showed a moderate expression of it in the cells of the valve petals.

OPN expression was strongly expressed in most CAV cells and extracellular space. Intense deposition of this protein in calcification sites was also noted.

Immunohistochemical study of AV samples of control groups showed the following results. During identification of $CD68^+$ cells in AV tissue, their number was found to be significantly lower than that of the control group ($p < 0.05$). On the other hand, CAV also contained a significantly higher number of MPO^+ cells ($p < 0.001$).

In the study of the expression of the S100A8/S100A9 heterodimer using fluorescent antibodies, significantly fewer cases of co-localization of these proteins as a single complex revealed than in the CAV group (Fig. 2.9). However, the expression level of S100A8 in the tissues of the control group was expressed moderately, mainly in the interstitial cells and was not significantly different from the group of samples of biomineralized valves ($p> 0.05$). Control groups showed markedly lower levels of S100A9 expression both in the cellular component of the tissue and in the extracellular space ($p < 0.01$).

This also concerned the expression of the Casp3 apoptosis marker ($p < 0.05$) and the OPN mineralization marker ($p < 0.001$), the levels of which were significantly lower in the control group.

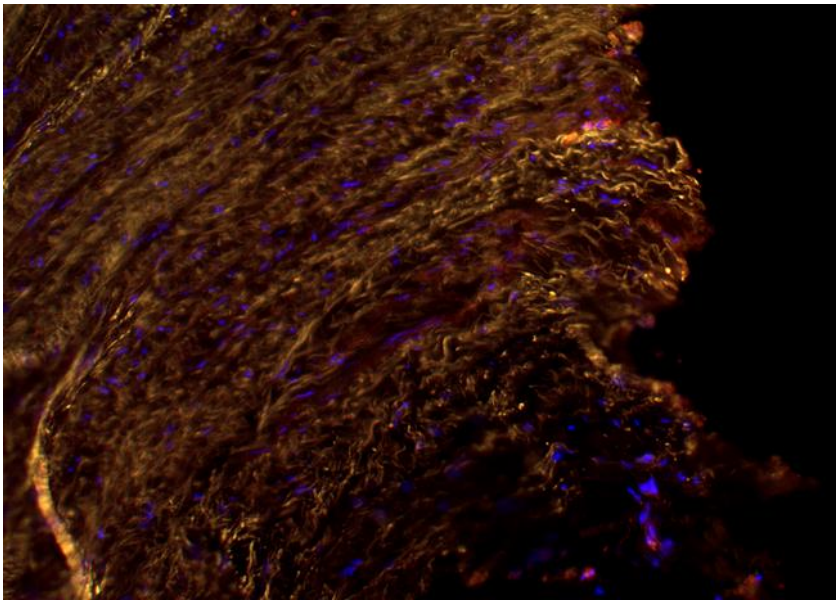

Figure 2.9 – Immunofluorescence study of S100A8 and S100A9 expression in group II aortic tissue co-localized as calprotectin at the border of the atheromatous plaque. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8/A9. Magnification x100

Therefore, in comparison with the results of immunohistochemical study of samples of aortic tissue in control group there is a noticeable difference in the expression of calprotectin complex: the content of its smaller subunit - calgranulin A was not statistically different from the control group of AV's. It is also necessary to note the difference in the authenticity level ($p = 0.42$) between $CD68^+$ cells in the groups studied. It can illustrate different conditions of development of pathological process at localization in the aorta and aortic valves: different expressiveness of inflammation, different degree of involvement in the atherosclerotic process (influence

on infiltrate composition), different local conditions (level of blood pressure, structural features). These causes affect the composition of donor cells of calgranulins. They ultimately cause differences in the features of the mechanism of development of pathological biomineralization. Obviously, this led to a predominance of the effect of calgranulin B in the tissue of mineralized AV. This necessitated further verification of the difference in the content of the key protein S100A9 in mineralized and control AVs. It was isolated from the valve tissue and quantified using dot-blot analysis.

The results of immuno-dot studies of S100A9 protein in AV tissue also showed a significant difference in its content in mineralized and control samples ($p < 0.05$).

The difference between the results of immunohistochemical study of MPO, CD68, S100A9, Casp3 and OPN expression in the 1st and 2nd groups of AV samples indicates their role in the processes of biomineral deposit formation.

Pathological biomineralization of heart valves leads to deep morphofunctional restructuring of all components of their structures. The quantitative and qualitative composition of the valve interstitial cells (VICs) and the fibrillar component (collagen and elastin) changes and due to that adversely affected endothelial cells (ECs) that cover the surface and interact closely with the interstitial valve cells, ensuring the integrity of the valve tissue [58]. Necessary conditions for the destruction of EC are created in atherosclerosis: infiltration by lipids, plaque and detritus formation, infiltration by circulating inflammatory cells (CIC) [124]. The CPCs (macrophages, lymphocytes, neutrophils, plasmocytes) are known to actively secrete calgranulins A and B, which make up the calprotectin complex (heterodimer) S100A8 / S100A9. It has cytotoxic effects at high concentrations [125].

In the study, the presence of calprotectin in AV tissue was confirmed by the co-localization of these proteins with their simultaneous staining with fluorescent antibodies against S100A8 and S100A9. A high concentration of S100A9, the active component of calprotectin S100A8 / S100A9, in tissues CAV was also detected using a dot-blot.

In conditions of inflammation, such influence of S100A8/S100A9 triggers apoptosis processes in EC and VICs (involving the mitochondrial pathway and caspase-3 and caspase-9), and necrosis may also occur [125, 126]. In our study, this was reflected in high Caspase 3 expression in the AV sample group with biomineralization compared to the control group ($p < 0.01$). Morphologically, this was manifested by desquamation and detachment of the endothelium, deep damage to the valve petals. Therefore, calprotectin-dependent EC damage may be a critical element in the morphogenesis of vascular biomineralization in chronic inflammation and atherosclerosis.

Much of the effects of S100A8 / S100A9 are realized through Toll-like receptors, which are present in EC, fibroblasts, and inflammatory cells [127, 128]. Activation of glycolysis end-product receptors (RAGE) and TLR4 can accelerate vascular calcification both in vitro and in vivo [129].

Several recent studies have been devoted to the study of OPN expression in cardiac valves [130, 131]. The results of our study show an increased expression of OPN in the tissue of mineralized heart valves - both in the extracellular environment and in the valve cells (interstitial and circulating) compared with the control group. Also, a significant amount of OPN was found in biomineral deposits. This is consistent with numerous publications that report that OPN binds to the surface of hydroxyapatite crystals, blocking its growth [118].

Osteopontin and calgranulin B are actually synthesized and secreted by the same cells, the leading role among which are

neutrophils and macrophages. In IHC study $CD68^+$ cells were found to be superior (in the authenticity level $p = 0.42$) in mineralized AV tissues ($p < 0.05$). The number of neutrophils in CAV also significantly exceeded the corresponding indicator of the control group. In fact, this means that calgranulin B-cytotoxicity and AV tissue damage are realized through neutrophils and cells of macrophage row. As calgranulins A and B make up about 40 % of the cytoplasmic proteins of neutrophils, this is logically consistent with the high concentration of calgranulin B in CAV [126] (Fig. 2.10).

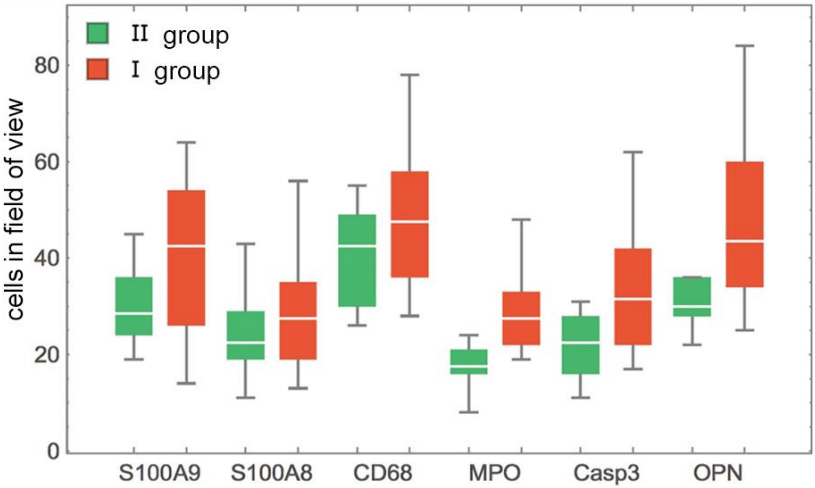

Figure 2.10 – Results of the immunohistochemical study of the tissue of the CAV cusps. * – statistically significant values ($p < 0.05$)

The inflammatory process in the AK leads to changes in the extracellular matrix of their tissues, disturbing the balance between local pro- and anti-calcium mechanisms. This creates favorable conditions for the deposition of minerals. For example, type I collagen in tissue CAV maintains osteogenic differentiation of SMCs, the number of which increases under

pathological conditions in valves from 5 % to 30 % [120]. Valve interstitial cells (VICs) that interact with collagen type I remain in a "calm" state for a long time. But with the appearance of fibrin, which inevitably occurs during the development of atherosclerotic plaque, they demonstrate a myofibroblastic phenotype and rapidly form calcium aggregates [120].

Therefore, under the influence of damaging factors, the desquamation of the integumentary endothelium and the denudation of the connective tissue matrix emerge. Cell death promotes the release of a large amount of inorganic phosphate and calcium. Thus, a considerable amount of building material and the presence of connective tissue matrix (type I collagen) creates conditions for the development of pathological biomineralization in human aortic valves, the mechanism of development of which can be demonstrated as follows (Fig. 2.11).

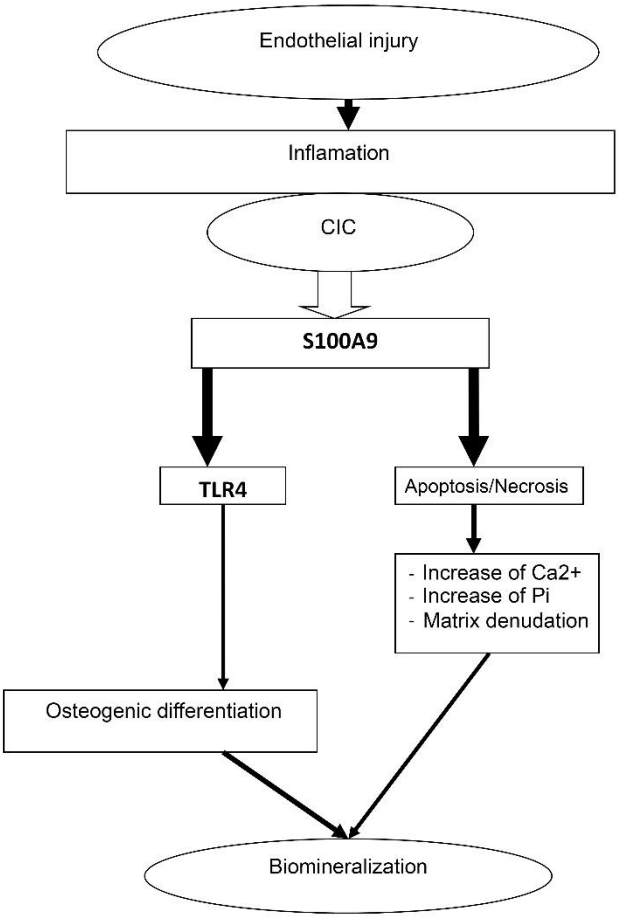

Figure 2.11 – A potential mechanism for the involvement of calgranulin B in the development of calcification AV

2.2 Pathological biomineralization in thyroid cancer

The share of thyroid cancer in recent decades has been steadily increasing worldwide. Thus, according to the worldwide study of the incidence and mortality of malignant tumors GLOBOSCAN in 2012 revealed 298 thousand new cases

of thyroid cancer. The highest incidence of thyroid cancer is observed in the United States (20 per 100 000 population among women and 6.3 per 100 000 among men). For Ukraine, the incidence of thyroid cancer has increased by 6-7 % annually between 1989 and 2013 [132]. the region has one of the highest rates of cancer incidence in the country, which in the last 5 years has fluctuated within 10.3 - 15.1 cases per 100 thousand population [41].

The decisive influence on the clinical behavior of malignant tumors of any localization and the overall survival rate of patients exerts a histological phenotype of the neoplasm, so the question of accurate diagnosis is extremely important. In recent years, a number of scientific papers have underlined the significant diagnostic and prognostic role of pathological biomineralization in malignant thyroid tumors (MTT) morphogenesis [133-135].

An important and poorly researched manifestation of pathological biomineralization in MTT tissue is vascular wall damage or vascular calcification (VC). This phenomenon is most often found as a manifestation of pathological biomineralization of the thyroid gland, rarely - follicular cancer of the thyroid gland.

Because the PTC is the most common thyroid tumor, occupying up to ¾ of the volume in the thyroid disease structure [38, 41], therefore, an extensive immunohistochemical study of vascular calcification of the thyroid gland was performed on a group of papillary thyroid carcinoma specimens.

The study of VC problems also covered the calcification of intravascular tumor emboli, which is a substrate for the formation of psammoma bodies and an integral part of pathological biomineralization of vessels [40].

Macroscopic examination of tissue specimens of follicular cancer of the thyroid gland showed the tumor as nodes, which often had a thick capsule in their composition. Biomineral

formations of follicular cancer of the thyroid gland are more often found during the dissection of tumor tissue than during visual examination of a malignant neoplasm. The incision was difficult and was accompanied by a characteristic crunch. The follicular cancer of the thyroid gland had a yellowish-grey color, relatively soft in consistency (Fig. 2.12A). Deposits of biominerals are most often localized in the tumor parenchyma and capsule nodes. They appeared in the form of solid formations of white color irregular shape, mainly had small sizes (up to 1.0 cm).

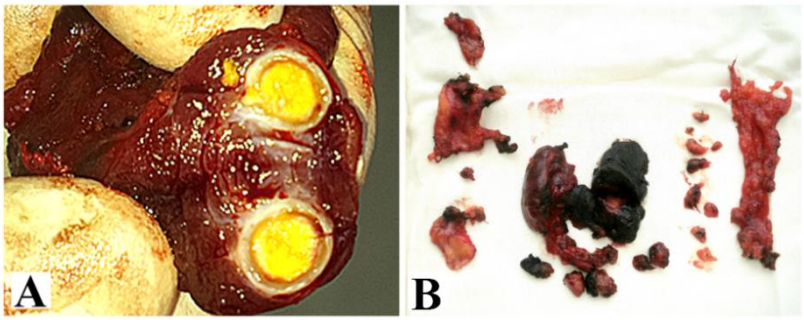

Figure 2.12 – Macroscopic examination of thyroid cancer. A. Macrophotography of follicular thyroid cancer. A thick solid white capsule is noticeable in the section. B. Macropreparation of papillary thyroid cancer with isolated lymph nodes and subcutaneous fat. The incision shows the tumor as a thick whitish tissue

Macroscopic examination of the PTC in most cases revealed a tumor of the nodular structure, with the presence of villi. In some cases, PTC was represented by cystic formation with bloody content and smooth papillary growths. The presence of a capsule for PTC was not a permanent feature. In general, the tumor tissue was white-grey, sometimes pinkish-yellow in color, with a dense texture (Fig. 2.12 B). The size of the tumor nodes varied from 0.8 to 4.0 cm. PBM of the PTC was

manifested as small very solid deposits of grey-white tissue, which were localized in the tumor parenchyma. In some cases, biomineralized formations were found in the pathomorphological examination of the lymph nodes.

In the course of histological examination of the follicular cancer of the thyroid gland, it was revealed that the tumors consisted of small follicles that were partially filled with colloids and formed by cells with varying degrees of atypism. Follicular cancer of the thyroid gland was characterized by invasive tumor growth into the surrounding tissue or capsule. Individual tumors consisted of solid masses of polygonal or spindle-shaped cells, with trabecular structure. Polymorphism, pathological mitoses were characteristic of the tumor cells of the follicular cancer of the thyroid gland, but such phenomena as nuclear grooves, accumulation of vitreous nuclei ("Orphan Ann's eyes", "egg basket") and psammoma bodies were not characteristic. The accumulation of condensed, bright pink colloid color in the follicles of adjacent healthy tissue was revealed by the staining of tumor tissue by PAS reaction (Fig. 2.13 B).

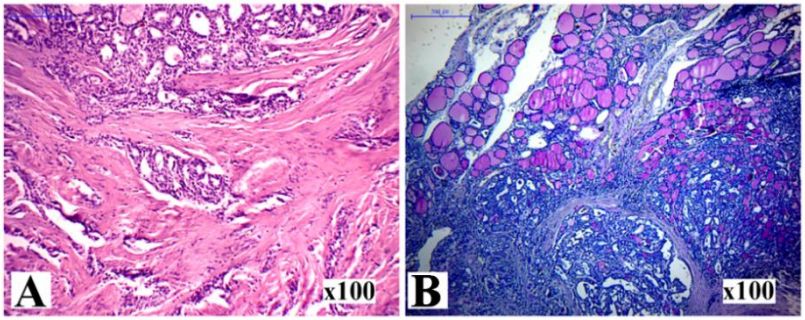

Figure 2.13 – Histological examination of the follicular cancer of the thyroid gland. A. Invasive growth of follicular cancer of the thyroid gland into the surrounding tissue. Hematoxylin-eosin staining. x100. B. Accumulation of PAS-positive colloid in thyroid tissues adjacent to the tumor. PAS reaction. Magnification x100

The processes of pathological biomineralization in the tissue of follicular cancer of the thyroid gland have damaged both the elements of the parenchyma (follicular epithelium, colloid) and the stromal component (capsule, interfollicular and interstitial layers). There was also a combination of biomineralization of parenchymatous and stromal elements with the formation of large margins of mineralized tissue, which were easily detected during the preparation of the material for histological examination (Fig. 2.14).

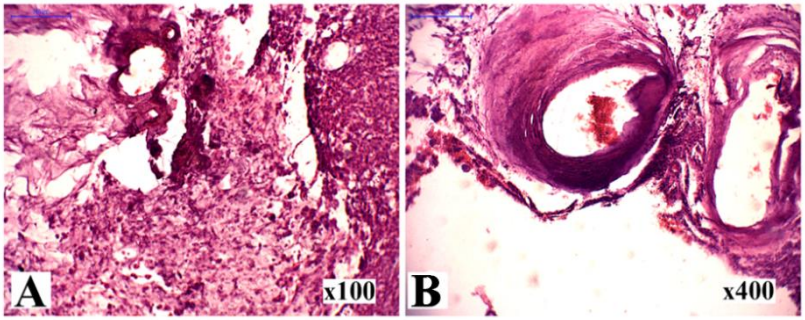

Figure 2.14 – Histological examination of the follicular cancer of the thyroid gland. A. Combination of biomineralization of stromal and parenchymatous tumor structures. B. Pathological biomineralization of venous and arterial vessels of the follicular cancer of the thyroid gland. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

It should also be noted that the phenomena of vascular calcification developed in vessels of medium and small calibre, mainly venous (Fig. 2.14 B, Fig. 2.15 A, B). Sometimes monolithic crystals in the form of boulders or plates were formed in the walls of vessels. The deposition of biominerals occurred in the inner and middle layers of the vessel walls, whereby predominantly partial calcification of the cross-section of the vessel in the limited length section was revealed. For small vessels (by type of capillaries, venules or arterioles),

characteristic total mineralization of the entire wall was observed.

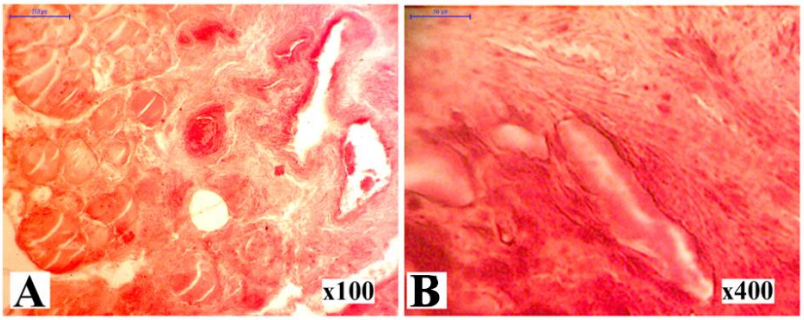

Figure 2.15 – Histological examination of vascular calcification of the follicular cancer of the thyroid gland. A. Biomineralization of vessel walls and stromal calcification. Alizarin red staining, mag. x100; B. Deposition of calcifications in the capsule and the walls of its vessels. Alizarin red staining, magnification x400

Histological examination of the PTC showed that the group of test specimens of thyroid neoplasms consisted of 21 cases of the classic version of the PTC (CvPTC) and 3 cases of the diffuse-sclerotic variant of the PTC (DSvPTC). In the histologic examination, CvPTC was a tumor of the papillary or cystic structure with the papillae that were branching. The papillae were put by one or more layers of epithelial tumor cells with signs of atypia. Highly differentiated tumors with monomorphic cubic epithelium were more common. There was a different degree of loss of differentiation with the development of cell polymorphism. The papillary outgrowths had a fibrotic base with a large number of dilated small vessels, small hemorrhages (Fig. 2.16 A). In addition to the vascular component, the connective tissue base of the papillae contained a marked inflammatory infiltration of mixed-cellular nature. A

high level of desquamation of the papilla epithelium was observed (Fig. 2.16 B). Pathological biomineralization, which accompanied CvPTC, was characterized by the presence of PBs, which were most often localized in the fibrous basis of the papillae (Fig. 2.17 A, B).

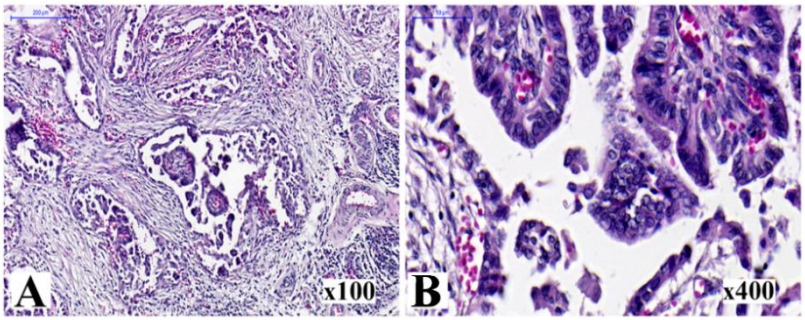

Figure 2.16 – Histological examination of the PTC. A. The classic version of PTC. B. Desquamation of the tumor epithelium into the lumen of cystic formations. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

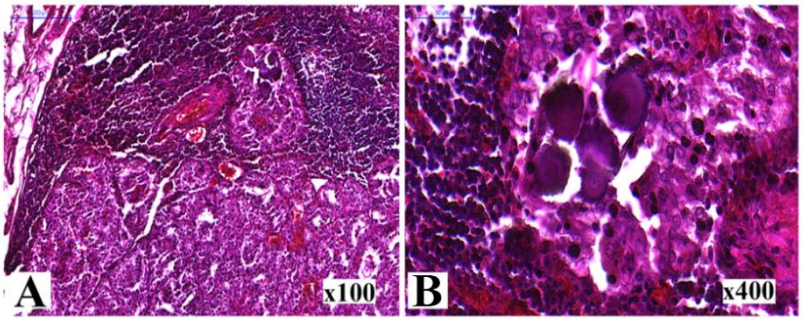

Figure 2.17 – Histological examination of the PTC. A. The classic version of PTC. B. Pathologic biomineralization of the PTC in the form of psammoma bodies. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

In the macroscopic examination, DSvPTC had the appearance of solid scar tissue of whitish-grey color. It is

expected that in the histological examination this variant of papillary thyroid cancer was characterized by a considerable amount of connective tissue and constant signs of pathological biomineralization in the form of various forms, especially PB (Fig. 2.18 A, B).

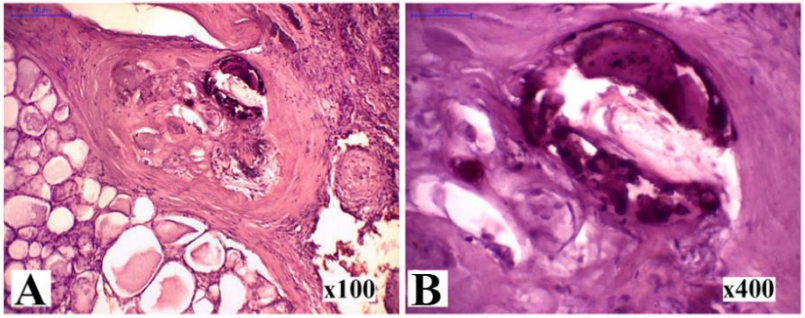

Figure 2.18 – Histological examination of the DSvPTC. A. The diffuse-sclerotic variant of PTC. B. Formation of psammoma bodies. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

The main manifestation of PBM in the tissue of the PTC was the formation of PB. They were manifested as rounded mineral objects consisting of concentric layers. Today, it is generally accepted that the formation of PT from tumor emboli, which under the influence of protective mechanisms of the body are subject to step biomineralization [39, 40, 55]. This was confirmed in histological studies, where the association of PT with the vascular component was constantly monitored (Fig. 2.19 A, B). Additional evidence of biomineralization of tumor emboli and their vasculogenic origin was the detection of PT in the collector lymph nodes and distant organ locations where they were also associated with vessels (Fig. 2.20 A, B).

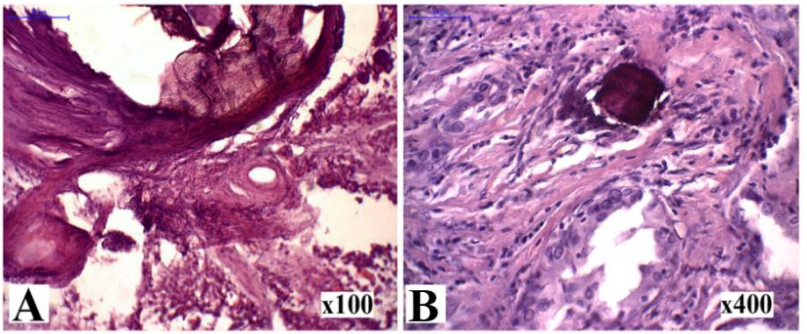

Figure 2.19 – Histological examination of the PTC. Relationship of PB with blood vessels. A. The diffuse-sclerotic variant of PTC. B. Formation of psammoma bodies. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

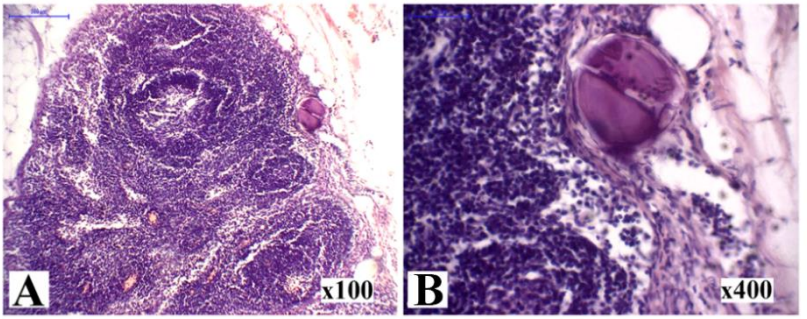

Figure 2.20 – Histological examination of the PTC. Relationship of PB with blood vessels. A. PB in the subcapsular region of the lymph node. B. PB in a vascular bed. Hematoxylin-eosin staining. Magnification in x100 and x400 respectively

In general, PBs had predominantly intra-tumor localization. Most often, it is the stroma of the tumor papillae, subcapsular areas, and tumor capsule. There were also an infrequent and extracorporeal location of PBs in the connective

tissue between follicles or lobules, intact thyroid lobe, and other organs, consistent with the notion of an "embolization" theory.

In order to confirm the presence of calcium compounds in the PB PTC, staining with alizarin red according to McGee was performed (Fig. 2.21 A, B).

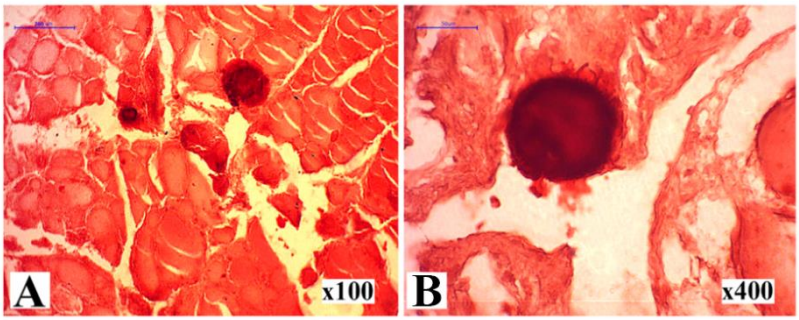

Figure 2.21 – Histological examination of the PTC. A. Positive response to Ca^{2+} compounds in the composition of PB. Magnification x100. B. PB with a "calcium background". Alizarin red staining, magnification x400

Vascular calcification in the tissue of the PTC is a more common pathohistological phenomenon in comparison with the follicular cancer of the thyroid gland. In the PTC, lesions of all types of vessels occurred: small - capillary type, medium - veins and arteries and large (Fig. 2.22). It is also interesting to observe the "contact transfer" of biomineralization from the PB to the vessel wall.

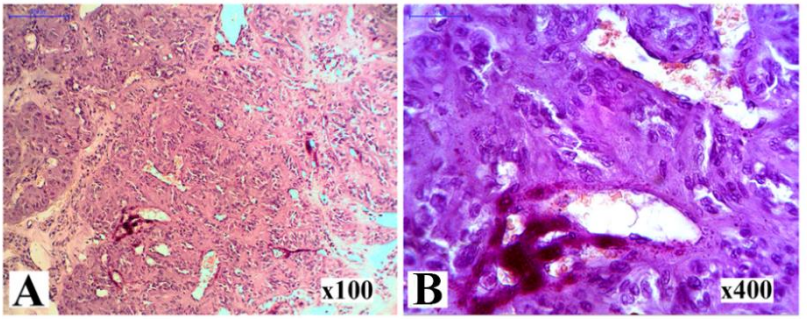

Figure 2.22 – Pathological mineralization in the vessels of the PTC. A. Biomineralization of small intra-tumor vessels. Magnification x100. B. Biomineralization of the capillary vessel. Magnifiation x 400. Staining with hematoxylin-eosin

Larger vessels underwent only partial biomineralization, which was limited to the inner or middle layer of the wall (Fig. 2.23). With the defeat of the PBM of the large arteries, the atherosclerotic origin of the pathology cannot be ruled out. For small vessels undergoing biomineralization in the tumor parenchyma, this process may be the mechanism of elimination ("shutdown").

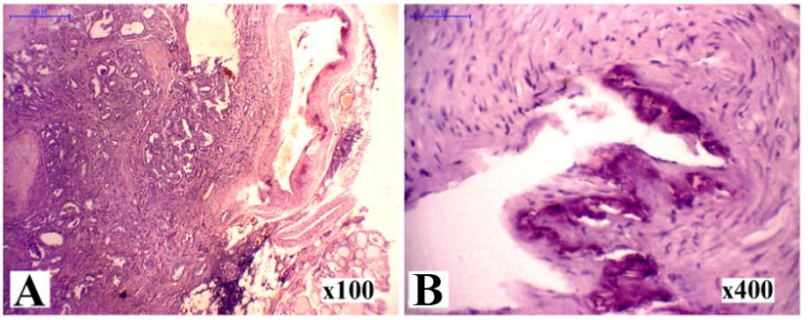

Figure 2.23 – Pathological biomineralization in the vessels of the PTC. A. The lesion of a large arterial vessel. Magnification x40. B. Biomineralization of the inner and middle layer of the artery. Magnifiation. x400

Thus, the major type of pathologic biomineralization of the PTC is vascular-dependent calcification, including psammoma bodies and vascular calcification. The staining of biomineralized objects with alizarin red indicates that they are composed of calcium compounds and they are able for reverse diffusion.

Morphological and physicochemical analysis of the mineral constituent tissue of MTT

For MTT biominerals, the content of calcium compounds was verified in stages: first using histochemical staining methods with alizarin red and von Koss stain, then scanning by electron microscopes, including microanalysis (EDX), were used. Further clarification of the presence of Ca^{2+} ions was carried out by the methods of applied materials science - X-ray diffraction (XRD), infrared spectroscopy (IRS), transmission electron microscopy (TEM) and X-ray spectroscopy (XPS).

Sample preparation for SEM was carried out by a modified technique, which made it possible to carefully separate the inorganic component from the residues of organic matter, while maintaining their relative structural integrity. This was especially important for the study of pathological biomineralization of vessels, where it is difficult to preserve the complex architecture of biominerals.

Thus, in the course of SEM complex fragments of mineral substance in the form of prints of vessels and their walls were revealed (Fig. 2.24 A, B). Obviously, the retention of the form was due to the close adherence to the soft tissues or by the mineralization of the organic matrix. The fragment of the vessel in the form of a semi-cylinder had an uneven inner surface, which can be compared with the "pavement" (Fig. 2.24 A). The outer surface of the vessel fragment was smooth, and the thickness of this curved plate ranged from a relatively narrow range of 10–20 μm. The morphology of the biomineral fragment

of the vessel was consistent with the histological structure of small vessels of the microcirculatory bed by capillary type.

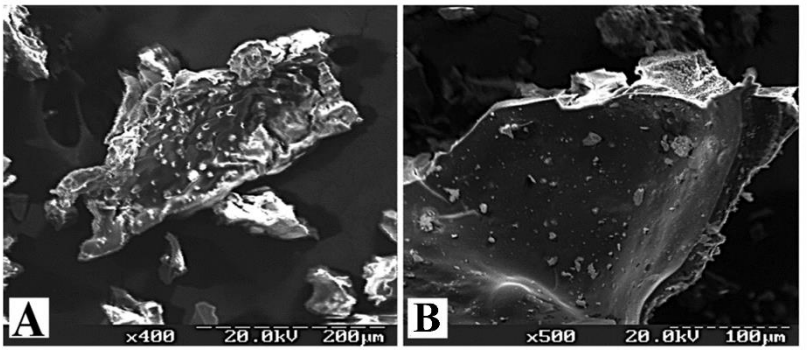

Figure 2.24 – SEM of biomineral deposits of the follicular cancer of the thyroid gland after low-temperature combustion (200 °C). A. Biomineral "sample" from a small vessel. B. Fragment of the vessel wall. The magnification and the marker are indicated at the bottom of the microphoto

Vascular calcification of MTTs (biomineralized vessels and PBs) were analyzed using SEM/EDX (Fig. 2.24–2.25). In electronic scanners, mineralized elements are manifested as bright white-grey objects with signs of destruction in the form of fragmentation and cracks, which are related to the peculiarities of sample preparation and their cutting at the microtome.

The morphology of PB has been described by us in previous works [36, 37]. In this study, PB focuses on the relationship and association of these objects with vessels. At SEM of the tissue of PTC it was revealed, that PBs were in vascular beds of different localization: stroma of papillae, capsule, interparticle and interfollicular connective tissue. During the passage of the microtomic slice through the tissue of mineralized PTC fragments of PB and biomineralized vessels of different morphology were revealed, which is mainly caused by

the position of calcified tumor blood clots in the vessels. In cross-sections passing through PBs and their blood vessels visible laminarity (lamination) structure. In the case of a microtome knife, structures in the form of cylinders or cork formations were found in the longitudinal plane of the biomineralized object. However, in most cases the sections were cut at different angles, which led to the destruction and decomposition of biomineralized objects (Fig. 2.25 A, B).

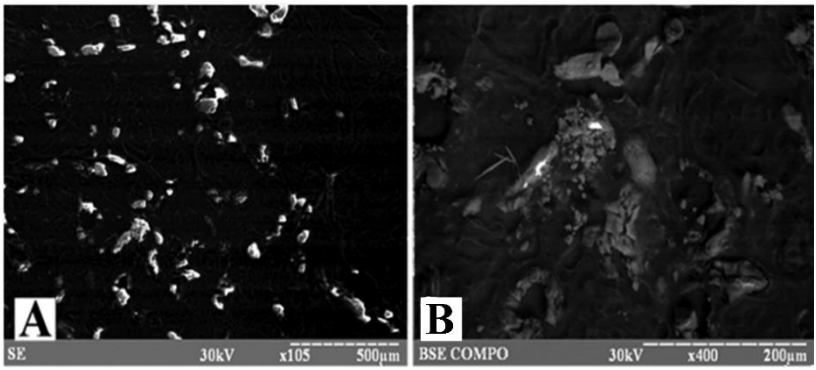

Figure 2.25 – Scanning electron microscopy of PTC specimens. A, B. Scans of numerous biomineralized vessels and PB in specimens of the thyroid gland. The scan mode, marker and magnification are indicated at the bottom of the microphoto

X-ray diffractograms of the mineralized components of the PB-vessel complexes showed a similar chemical composition and a high level of their mineralization and saturation with calcium and phosphorus (Fig. 2.26).

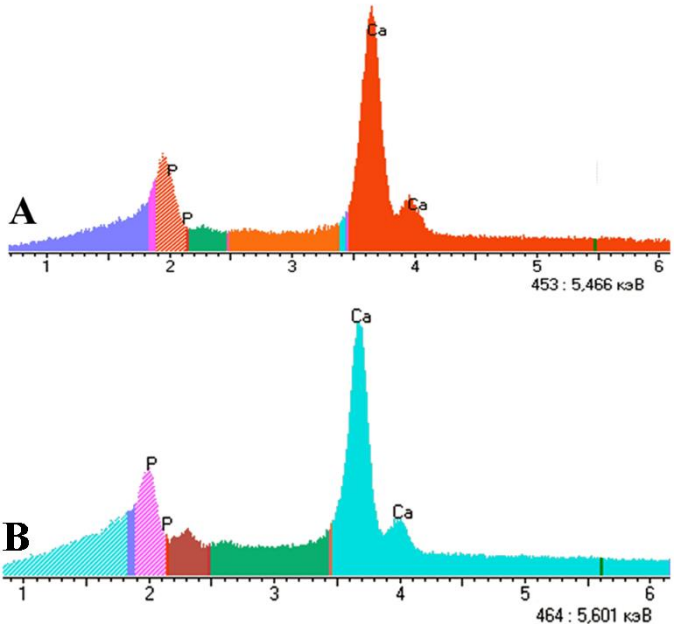

Figure 2.26 – X-ray diffraction spectra of biomineralized objects of PTC samples

In addition to the PBs and vessel walls associated with them, the SEM and microanalysis of the capsule vessels and interstitial stroma with signs of biomineralization were also performed. On the scanned scans the vessels had characteristic whitish-grey staining, which indicated the presence of biomineralization processes (Fig. 2.27 A, B). According to the microanalysis of the composition of the surface of the sample revealed a high content of calcium (about 40 %), which confirms the presence of its biominerals. The level of mineralization of these cases was lower than in PB.

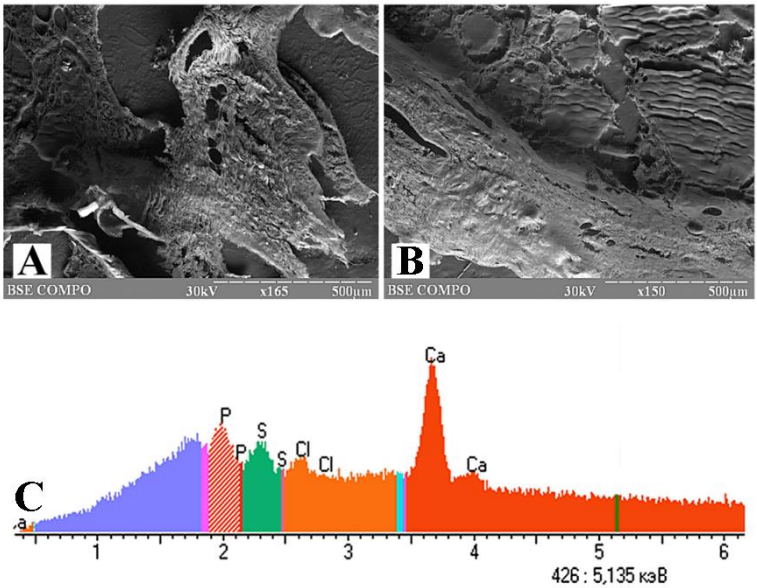

Figure 2.27 – X-ray scanning electron microscopy of the follicular cancer of the thyroid gland with X-ray microanalysis. A, B. Biomineralized vessels in the capsule and the inter-follicular stroma of the tumor. B. X-ray diffractogram of mineralized tissue. The green marker indicates the microanalysis sections. The magnification and marker are indicated in the lower right corner of the mircrophoto

According to diffraction methods (Fig. 2.28–2.29), the main crystalline phase of the MTC biominerals is well-structured hydroxyapatite, often with β-TCMP (beta-three-calcium-magnesium phosphate) impurities. In the EDX spectra of PTC, in addition to the basic lines Ca and P, weak lines S, K, Cl and some other elements are often present, which is typical for most biominerals of the human body. The intensity ratio of the Ca and P lines is close to that characteristic of apatite.

The IR spectroscopy data are in good agreement with the above structural analysis results and confirm the apatite nature of the biominerals (Fig. 2.30).

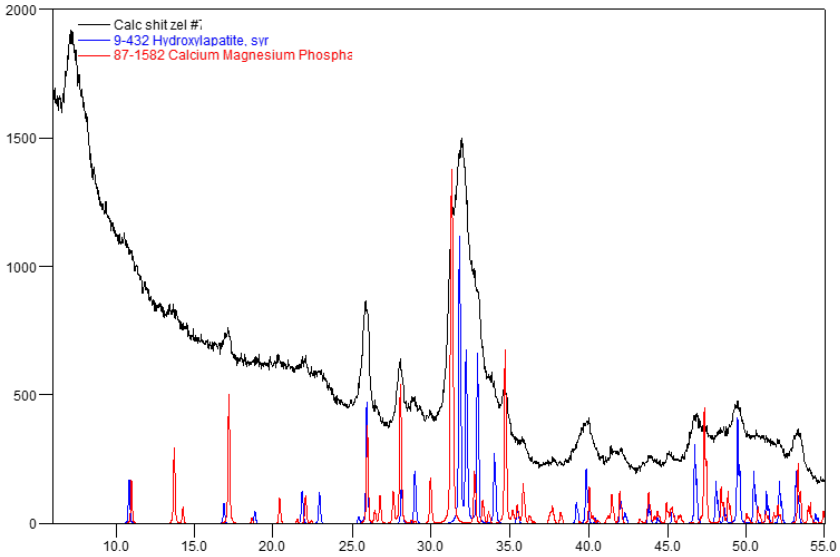

Figure 2.28 – X-ray diffractogram of the biomineral deposit of the PTC. The presence of apatite and β-TCMP are present in the biominerals

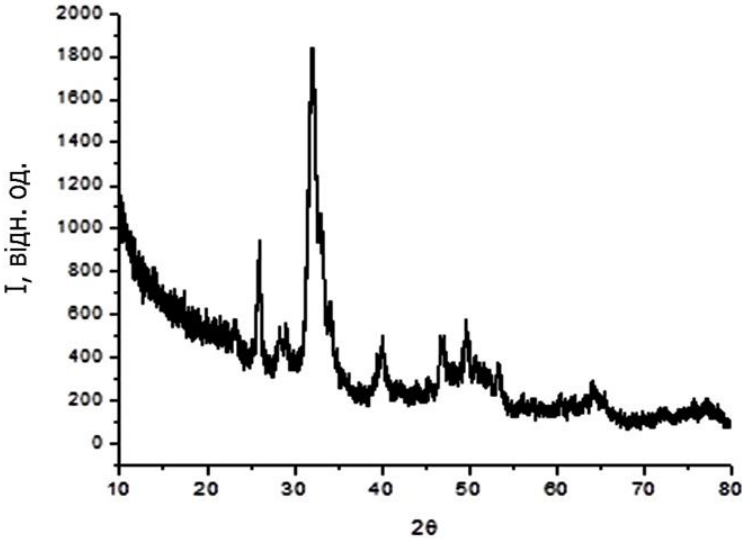

Figure 2.29 – X-ray diffraction pattern of the biomineral deposit of the PTC. Only structured hydroxyapatite is present in the biomineral

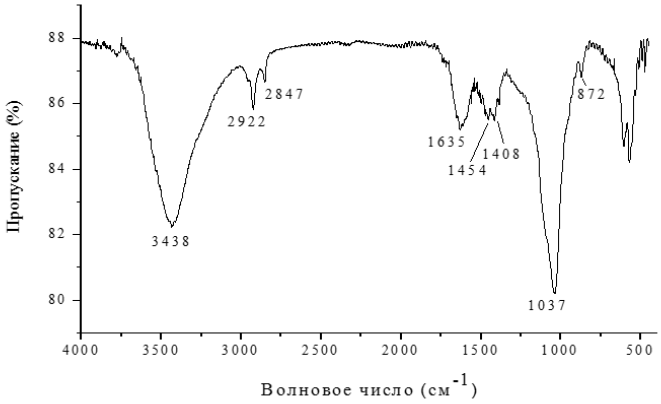

Figure 2.30 – The typical IR spectrum of pathological thyroid biominerals

The results of TEM and ED confirm the presence of apatite in all investigated thyroid deposits (Fig. 2.31). According to the TEM, apatite crystals can be of different dispersions and sizes (Fig. 2.31 A). The ED pattern shows the polycrystalline of pathological biominerals and the features of organization and texture of their composition (Fig. 2.31 B).

Thus, the presented data demonstrate a wide variety of sizes, shapes and orientations of calcified crystals of the thyroid gland. They confirm their phase attachment to calcium apatites.

Because the TEM and ED studies do not require a large amount of sample material (compared to X-ray diffraction), it has made it possible to analyze pathological biominerals of different sizes and localization. Also, the results of the study gave an opportunity to understand the structural and morphological features of biominerals in connection with their location in pathological formation or the thyroid gland as a whole.

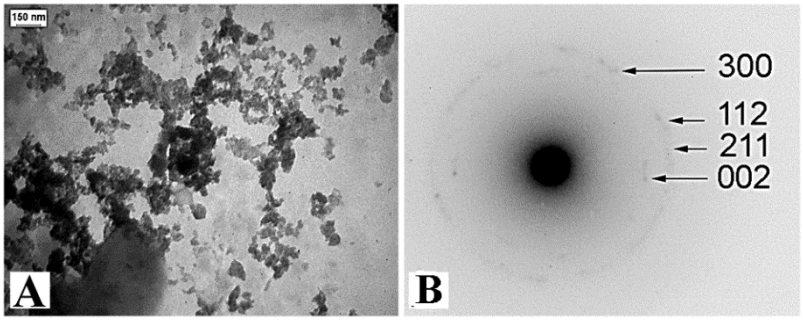

Figure 2.31 – Transmission electron microscopy of nanocrystals (A) and the ED pattern (B) of a sample of biomineral formation

Additionally, X-ray spectroscopy (XPS) was performed on specimens of PTC biominerals in which PBs were detected. The analysis of the composition of PB with XPS showed that PB consists of a large number of chemical elements, but with a

predominance of calcium, phosphorus, oxygen and hydrogen in the ratios corresponding to hydroxyapatite (Fig. 2.32). This is also confirmed by the number of studies described above.

A very important complement to the XPS results is the fact that the PB contains organic components that are of a protein nature: the presence of carbon, nitrogen, and oxygen directly indicates the presence of protein in the structure of the PB (Fig. 2.32 B, D). Evaluation of the results of X-ray spectroscopy on the content of calcium, phosphorus and phosphate group suggests that the content of hydroxyapatite in PB is approximately 80 %.

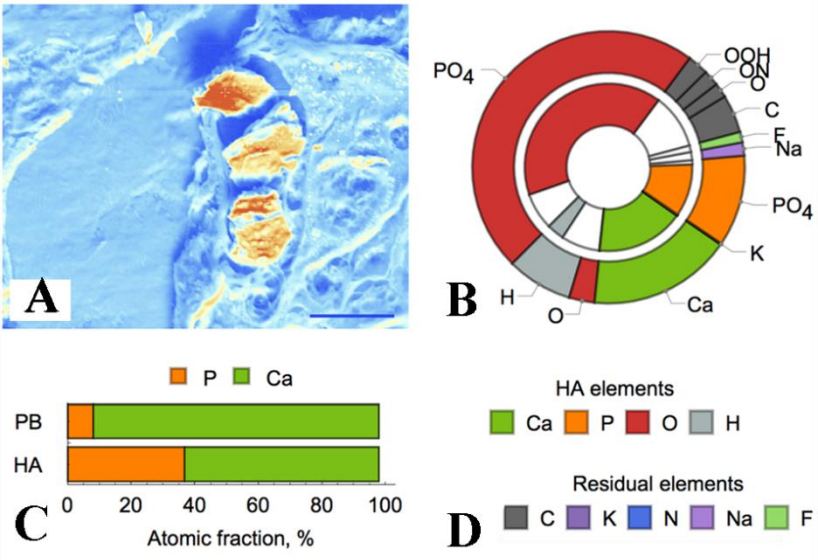

Figure 2.32 – The composition of PB based on XPS results. A. Artificially stained SEM PB for differentiation of constituent elements: blue-stained light elements, red-stained heavy elements. B. Chemical composition of PB on phosphorus and calcium fractions obtained from SEM microanalysis. B-D. Chemical composition of PB according to XPS results

Thus, the following examples of the existence in pathological deposits of calcium-phosphate crystalline formations of different morphology confirmed by their different mechanisms of morphogenesis. This development occurs in an inseparable interaction with the organic tissue, forming organic-mineral aggregates (OMA) of biocomposite type. To a large extent, the direction of development of biomineralization and its biological function are determined by the organic environment and the peculiarities of the pathological process that develops in soft tissues.

High regional incidence of thyroid cancer and other thyroid diseases in the Sumy region, and in general, in the North-Eastern region of Ukraine has long been the focus of attention of scientists [136–141]. The peak incidence of thyroid cancer in the Sumy region was in 2014–2016, reaching one of the highest levels in the world of 11.7–15.1 cases per 100 thousand population [41].

One of the first attempts to review the role of biomineralization in the morphogenesis of thyroid tumors was initiated as early as 2004 by Das, but these studies have not received the appropriate resonance [54]. However, in the last two years, there has been a movement towards rethinking many of the established stereotypes of classical thyroidology, especially the excessive, "hard" treatment of tumors with indolent course. This is due to the fact that the US alone spends $ 1.3 billion on the treatment of thyroid cancer and its effects [142]. In 2016, a multicenter study was published on the reclassification of some types of malignant tumors of the thyroid gland (encapsulated follicular variant of the PTC) to a benign condition.

In the same year, a study of Japanese scientists appeared about calcification and vascularization of the tumor site in the PTC. They found that tumors with intensive blood supply were statistically more often larger than tumors with reduced blood

supply. Therefore the conclusion is made about the relatively benign course of thyroid cancer with the phenomena of biomineralization [133].

Against this background, there is an increased interest to such a poorly researched phenomenon as the biomineralization of vascular walls in the tissue of MTC. Vascular calcification (VC) is more characteristic as a manifestation of pathological biomineralization of the PTC, rarely - the FTC. As already noted, under the conditions of the PTC, lesions of all types of vessels occur: small - capillary type, medium - veins and arteries and large. In addition, assessing PB as a manifestation of VC, the interaction of the tumor process and the body emerges in a new light. It becomes clear the protective orientation of the phenomenon of vascular calcification under the conditions of MTC. Probably, similar orientation has VC and at malignant tumors of other localizations.

Another extremely interesting observation is the so-called "contact transfer" of calcification from the PB to the vessel wall, which has been detected in several histological preparations. Developing this thought and taking into account our previous studies (formation of calcium "halo" around biomineralized objects when stained with alizarin red), we can assume the possibility of reverse diffusion of calcium ions from biomineral deposits, which would create the conditions for the spreading of calcification processes to the surrounding tissues.

VC has a wide variety of manifestations in its detailed study. Sometimes monolithic crystals in the form of boulders or plates are formed in the walls of vessels. The deposition of biominerals occurs in the inner and middle layers of the vessel walls, whereby predominantly partial calcification of the cross-section of the vessel in the limited length section was revealed. Small vessels (by the type of venules or arterioles capillaries) are characterized by total mineralization of the entire wall, as was seen on individual SEM microphotos. Biomineralization of

vessels of the microcirculatory bed of the tumor parenchyma may be a manifestation of the protective antitumor mechanism of the body, so it is a promising area of research in oncology.

Partial calcification of a large calibre vessel (artery) causes some debate regarding the similar localization of biomineral deposits in atherosclerotic lesions. However, despite the fact that biomineralization is limited to the inner or middle layer of the artery wall, the phenomena of lipid deposition or formation of atherosclerotic plaques are not observed.

Thus, the main types of pathological thyroid biomineralization are:

- vascular-dependent calcification, including psammoma bodies and vascular calcification, which is more common in MTC;

- stromal calcification, characteristic of the structural components of the thyroid gland (lesions of the capsule, interparticle and interfollicular connective tissue);

- parenchymal calcification, characteristic for the follicular epithelium and thyroid gland colloid (Fig. 2.33).

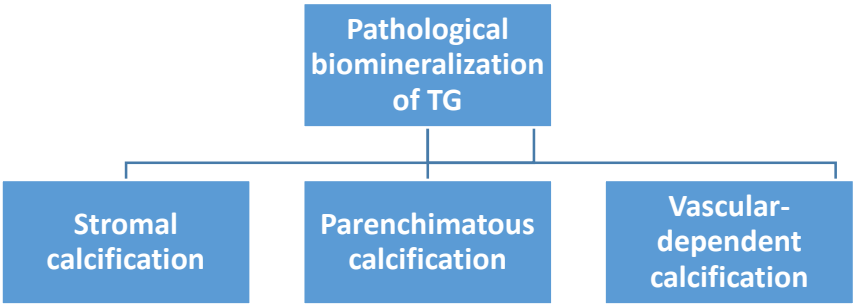

Figure 2.33 – The main types of pathological biomineralization in thyroid pathology

An immunohistochemical study of VC of MTT was performed on the material of the PTC specimens, as this tumor mostly characterized by vascular-dependent calcification. In

addition, the proportion of PTC is ¾ of the volume in the structure of thyroid disease [38, 41].

The study of VC problems also covered the calcification of intravascular tumor emboli, which is a substrate for the formation of psammoma bodies (PB) and also an integral part of the pathological biomineralization of vessels [40, 134].

Therefore, in the comparison of the results of immunohistochemical study of protein expression in PTC tissue with biomineralization (group I) and control samples of PTC without mineralization (group II), a significant difference was found for proteins - components of calprotectin complex - S100A8 ($S < 0.001$), Casp3 apoptosis markers ($p < 0.001$), OPN biomineralization markers ($p < 0.001$). For the marker of MPO neutrophils and cells of the CD68 macrophage row, no significant difference between the parameters of the studied groups was found ($p > 0.05$) (Fig. 2.34).

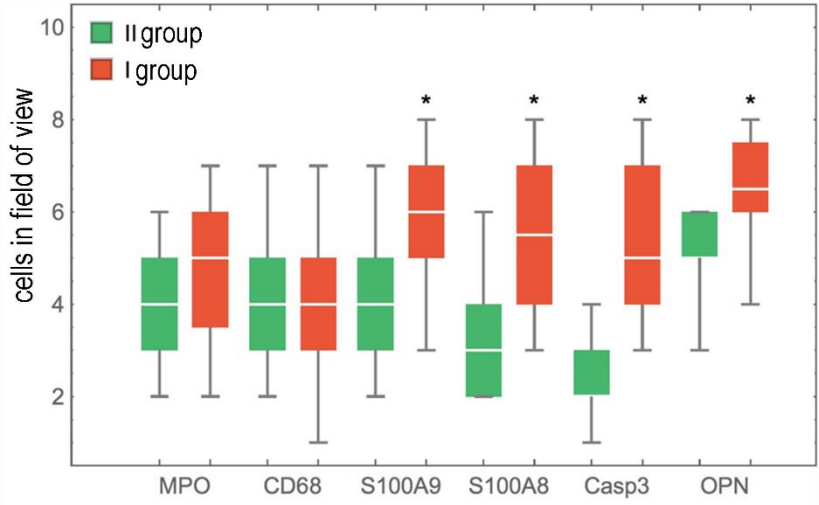

Figure 2.34 – Results of immunohistochemical study of PTC tissue. Evaluation of the intensity of the immunohistochemical reaction. * – statistically authentic values ($p < 0.05$)

Thus, the results of immunohistochemical studies for PTC have their own characteristics. The presence of significant inflammatory infiltration in the stroma of the papillae of both PTC groups offsets the difference between the results of these groups. Of particular note is the ability of PTC tumor cells to secrete OPN. Obviously, only additional stimulation of tumor microenvironment cells by biomineral deposits led to the preference for OPN expression of mineralized samples over the control group.

The expression of the components of the calprotectin complex (S100A8 and S100A9) and Casp3 were significantly higher in the group of mineralized PTC, which is consistent with the functional meaning of these proteins. It is noticeable that in the mineralized tissues of the PTC, the S100A8 and S100A9 proteins are more often co-localized in the cytoplasm of cells as part of the heterodimer calprotectin complex (Fig. 2.35). On the other hand, in the PTC control group, the frequency of protein expression coincidence is much lower. (Fig. 2.34). However, the question of the origin of more S100A8 and S100A9 in the mineralized tissues of the PTC remains unclear. If we analyze the results of immunohistochemical study (Fig. 2.35 and 2.36), we can note the expressed expression of calganulins by tumor cells.

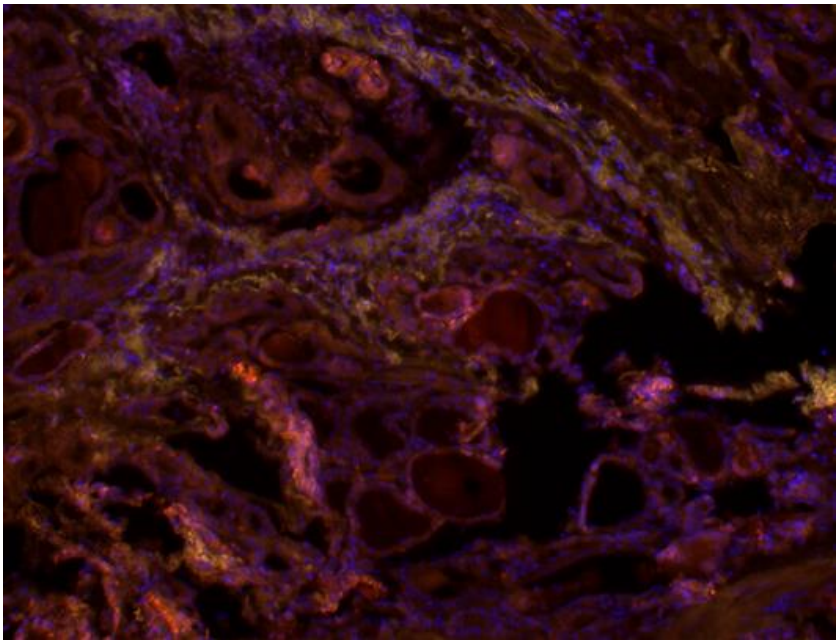

Figure 2.35 – Immunofluorescence study of the expression of S100A8 and S100A9 proteins in I group of PTC tissue, which co-localized as calprotectin. Blue is dapi, blue-green – Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x100

Perhaps cancer cells express calganulins to reduce the number of calcium ions in their microenvironment as a protective mechanism. This question remains open and may be a field for further research.

The presence of the difference between the results of immunohistochemical study of the expression of S100A8, S100A9, Casp3 and OPN in the 1st and 2nd groups of PTC testifies to their role in the processes of pathological biomineralization of vessels under conditions of malignant tumor process (Fig. 2.36).

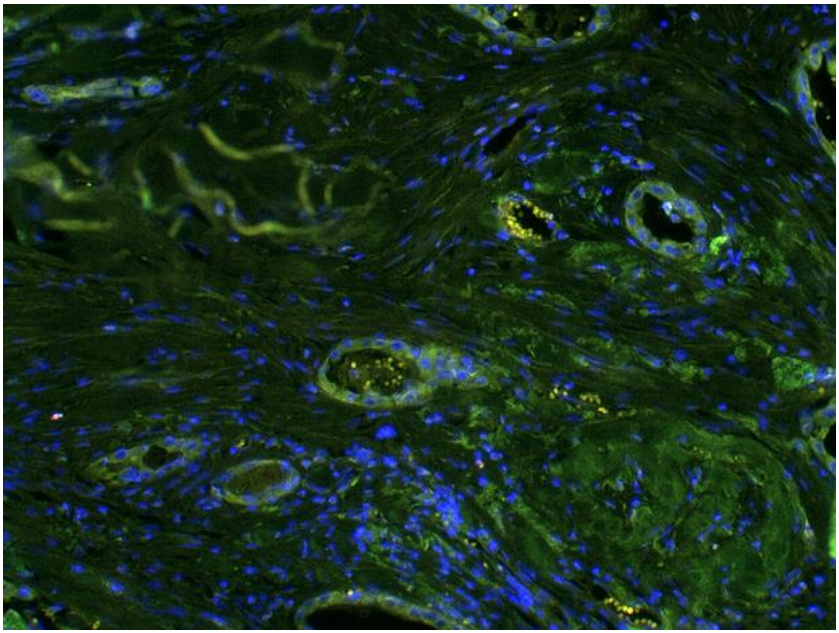

Figure 2.36 – Immunofluorescence study of S100A8 and S100A9 expression in II group of PTC tissue. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x100

The algorithm for the study of PBM by the methods of applied material science for MTC was the same as for the biomineral deposits of the cardiovascular system. Initially by using histochemical methods, namely by staining with alizarin red, identified the presence of Ca^{2+} ions, then scanning electron microscopy, including microanalysis (EDX), was applied. X-ray diffraction (XRD), infrared spectroscopy, transmission electron microscopy, and X-ray spectroscopy methods have finally completed the study of the biomineral phase of MTT calcifications.

SEM showed the presence of complex fragments of mineralized vessels and their walls (Fig. 2.25 A, B). The morphology of the biomineral fragment of the vessel was consistent with the histological structure of small vessels of the microcirculatory bed by capillary type.

When conducting SEM with microanalysis, it was found that the vessels of the capsule and interstitial stroma had a lower level of mineralization than the PB in combination with the remains of the vessels.

According to the diffraction methods (Fig. 2.29 and Fig. 2.30), it is revealed that the main crystalline phase of the MTC biominerals is well-structured hydroxyapatite, often with β-TCMP (beta-three-calcium-magnesium phosphate) impurities. In the EDX spectra of MTC, in addition to the basic lines Ca and P, weak lines S, K, Cl and some other elements are often present, which is typical for most biominerals of the human body. The intensity ratio of the Ca and P lines is close to that characteristic of apatite.

IR spectroscopy data are also consistent with the results of structural analysis and confirm the apatite nature of biominerals.

For the first time X-ray spectroscopy (XPS) was used to study PBM of MTC. For this purpose, samples containing only PB were selected. In addition to the fact that PBs consist of such chemical elements in ratios corresponding to hydroxyapatite (Fig. 2.32 A, B), the presence of organic components related to the protein nature was established. These data are completely new, as well as setting the hydroxyapatite content in PB at 80 %.

Thus, all cases of thyroid calcification in its most common malignant tumors corresponded to calcium-phosphate-type biomineralization. Structured hydroxyapatites with β-TCMP impurities were the main phase of biomineral deposits of MTC. Also, for the first time, the presence of organic matter residues in PB was detected by X-ray spectroscopy. This

indirectly confirms our assumptions about the role of the protein component (osteopontin) in the formation of the lamellar structure of PB.

2.3 Pathological biomineralization in the prostate

Most scientific papers dealing with the processes of pathological biomineralization in the prostate indicate that prostatoliths (prostatic lithiasis) are formed by dystrophic calcification of starch or amyloid bodies (Latin name – Corpora amylacea - CA), mainly formed in the lumen of glands of amyloid proteins [143]. This theory was started in 1861 when it was proposed by Sir Henry Thompson.

This view of the genesis of amyloid bodies and prostatoliths, as different stages of the same process, has many adherents among modern researchers [145]. According to Thompson's theory, desquamated epithelial cells give a start to the processes of concernments formation. They are nucleation cores on the basis of which the amyloid bodies are formed in the protein-rich prostatic fluid. It was believed that these little bodies acted as foreign bodies, which provoked an inflammatory response. Calcium ions and phosphates from degenerating epithelial cells were incorporated into amyloid bodies [144]. In general, the theory was accepted, only discussions were held on the mechanisms of CA calcification. Some authors guess the formation of prostatoliths by two related mechanisms. The main essence in both cases is obstruction and stagnation of the prostatic fluid [145]. Addition of the inflammatory response in conditions of stagnation of the secretion initiates and intensifies the processes of biomineralization [146].

Obtaining the most complete picture of the origin and maturation of prostatoliths is not possible without a detailed study of the concretions and surrounding soft tissues of the prostate. In addition, to understand the phase composition of deposits, the size and prevailing shape of crystals, the structural

and concentration features of the formed biominerals, it is necessary to study the conditions that lead to biomineralization. For this purpose, the prostate material with signs of diffuse benign prostate hyperplasia (BPH) without the presence of prostatic lithiasis (first group) and BPH with the presence of calcifications (second group) was investigated.

Both groups of BPH samples during histological examination were characterized by the presence of secretion, the phenomenon of stagnation, amyloid bodies in the enlightenment of the prostatic glands, and inflammatory infiltration around them. It should be noted that the predominance of these features was observed in the group of BPH samples with prostatic lithiasis.

Immunohistochemical study showed certainly higher expression in group I of prostate samples for markers of cells of the CD68 macrophage row ($p < 0.001$), components of calprotectin complex - calgranulins A (S100A8 - $p < 0.001$) and B (S100A9 - $p < 0.001$), markers of apoptosis Casp3 ($p < 0.01$) and OPN biomineralization marker ($p < 0.05$). For the MPO neutrophil marker, no significant difference between the groups studied was found ($p > 0.05$).

Despite the difference between the two groups compared, a relatively high level of inflammatory cell marker expression and apoptotic activity should be noted in the control group.

The number of S100A8- and S100A9-positive cells is also higher in prostate tissue with biomineral concrements, but compared to other mineralized tissues, the rate of formation of the calprotectin complex is lower (Figs. 2.37–2.38).

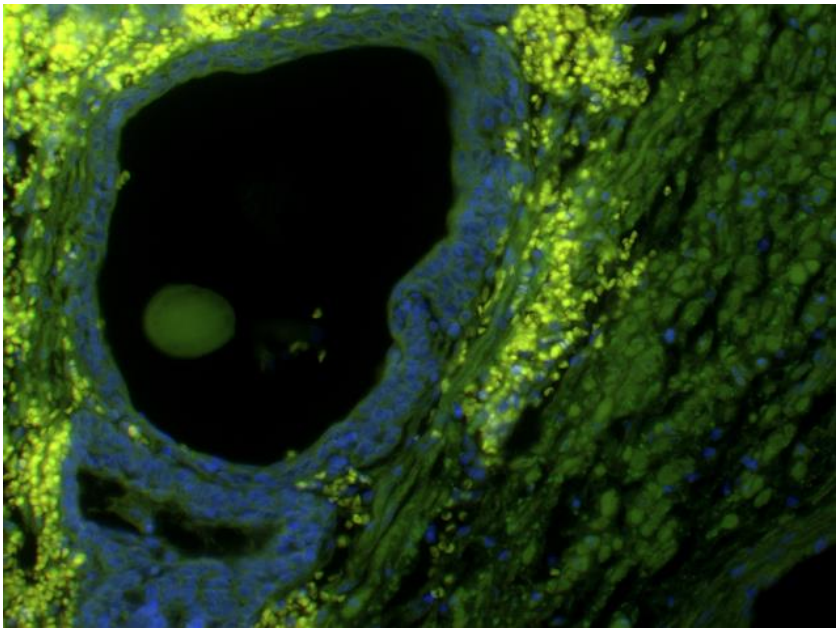

Figure 2.37 – Immunofluorescence study of S100A8 and S100A9 protein expression in group I of BPH tissue. Blue is dapi, blue-green - Alexa 488 (S100A8), yellow-green - Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x200

In a detailed study of the location of immunofluorescence antibodies against calgranulins A and B, it was observed that they are expressed separately or as part of the calprotectin complex in the papillary folds of the epithelium of the glands and in the lumen of small vessels (hemocapillaries). The presence of co-localization of S100A8 / S100A9 in prostatosomes, that separate from the apical part of epitheliocytes into the lumen of the glands, is constantly noted, forming a high intraluminal concentration of these proteins. This apparently results in the presence of calganulin B in prostatolite extracts, which was confirmed by Western blotting. Bands of

S100A9 protein were present in all specimens of prostate stones (Fig. 2.39). Another interesting phenomenon, which is often repeated, is a large number of calprotectin complex in the basal part of the cytoplasm of the prostate epithelium (Fig. 2.38 B). The value of the "basal accumulation" of calprotectin remains incomprehensible and needs an in-depth study.

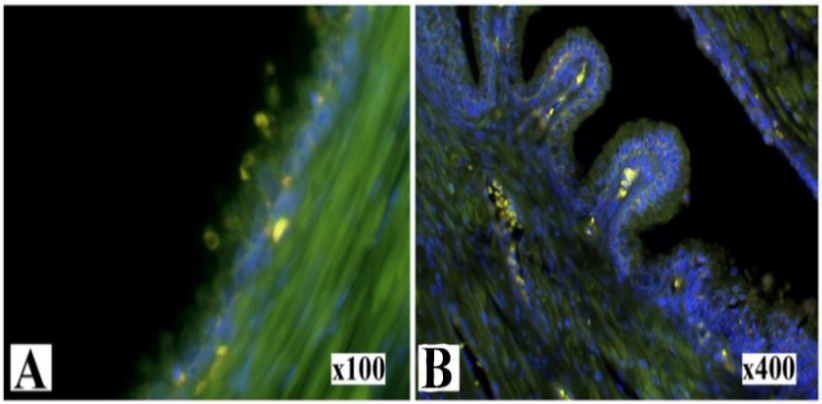

Figure 2.38 – Immunofluorescence study of S100A8 and S100A9 protein expression in group I of BPH tissue.
A. Prostatosomes containing calprotectin. Magnification x400.
B. S100A8- and S100A9-positive cells are present in the stroma of the papillary folds of the epithelium of the glands and in the capillaries. Magnification x200. Blue is dapi, blue-green – Alexa 488 (S100A8), yellow-green – Alexa 555 (S100A9), orange is calprotectin S100A8 / A9

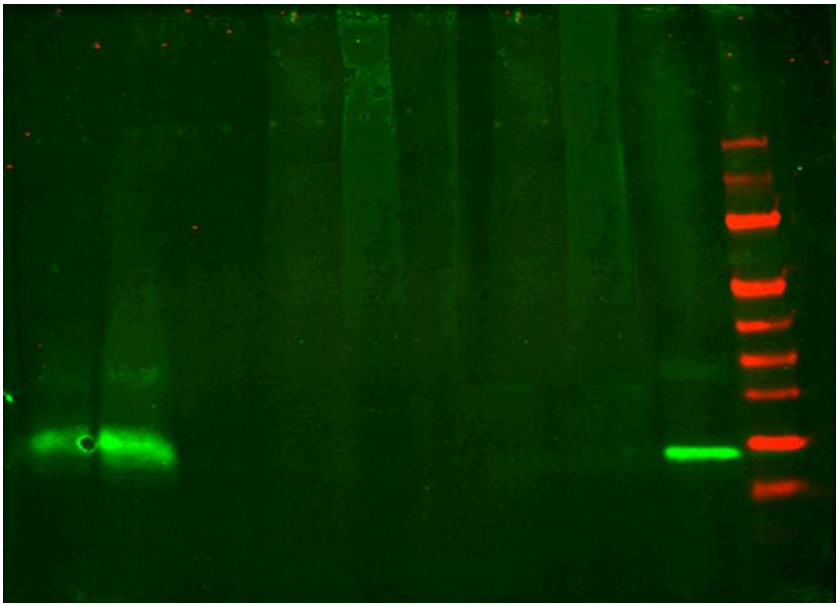

Figure 2.39 – Western blot of prostatolith extracts (1–2 lines) and amyloid bodies (3–7 lines) with luminescent antibodies against S100A9 protein. Line 8 – S100A9 Protein Control Solution. Molecular markers of proteins are shown in red on the membrane. Bands of calgranulin B are located in the lower part of the membrane in the region of 12–15 Kd

In the control group of BPH samples, the expression of S100A8 and S100A9 proteins was markedly lower, although it was also present at a stable level. The presence of these proteins in the enlightenment of the capillaries the basal parts of the epitheliocytes was also noted. The bright fluorescence of amyloid bodies was revealed, which is largely due to the autofluorescence of the proteins that make up its composition (Fig. 2.40).

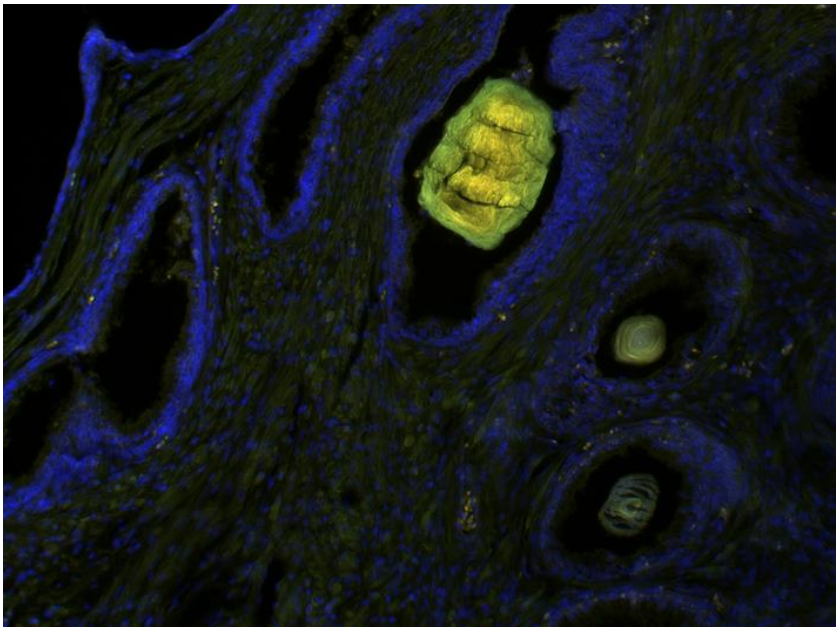

Figure 2.40 – Immunofluorescence study of S100A8 and S100A9 protein expression in group II of BPH tissue. Blue is dapi, blue-green – Alexa 488 (S100A8), yellow-green – Alexa 555 (S100A9), orange is calprotectin S100A8 / A9. Magnification x200

The fluorescence localization of calgranulins A and B in the prostate tissue was correlated with inflammatory infiltrate and circulating inflammatory cells (CICs) around the glands, in the small vessels lumen, and in the connective tissue component. It is also apparent that the presence of prostatoliths supports the inflammatory process and promotes desquamation of the prostatic epithelium (increased Casp3 expression), which is the source of building materials for biomineral deposits.

In addition to the fact that OPN is "attracted" by the presence of biomineral deposits in tissues (in this case –

prostatoliths), this protein is characterized by a certain level of expression in the inflammatory infiltrate [86, 118].

The difference between the results of the immunohistochemical study of the expression of CD68, S100A8, S100A9, Casp3 and OPN in the 1st and 2nd groups of BPH samples indicates their role in the processes of prostatoliths formation under benign prostatic hyperplasia.

According to the PEM data (Fig. 2.41 A), crystals of pathological prostate deposits have mostly irregularly rounded shape with average sizes of several tens of nanometers with a monomorphic composition of crystalline particles. The surface/volume ratio of prostate nanocrystals indicates a significant area of interaction with the organic component or biological fluid of the luminal section of the prostatic glands.

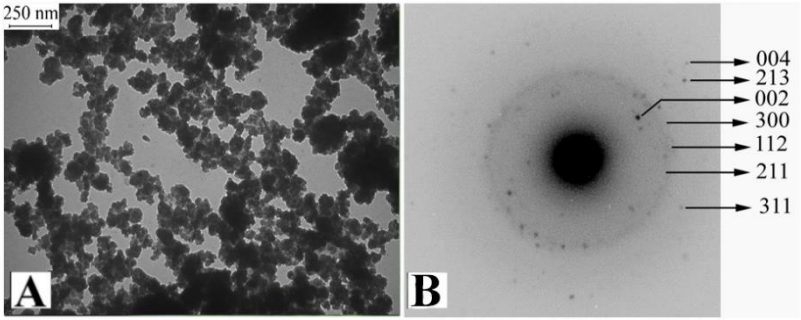

Figure 2.41 – Investigation of prostatoliths using TEM and ED. A. Electron microscopic images of crystalline prostatoliths particles. Magnification x40 500. B. Typical electron diffraction pattern on prostate calcite crystals. On the right – are the crystallographic indices of hkl apatite

In general, the beginning of the mechanism of formation of intraluminal inclusions of the prostate should start with age-related changes of the organ. Remodeling of the prostate tissue under the influence of age is characterized by hyperplasia of the fibro-muscular component, which leads to compression of the

glands and their ducts, complication of drainage of the secretion from the prostate, formation of the phenomena of stagnation. Retention of gland secretion or urine reflux into the tissue of the prostate in case of difficulty in urination, causes the development of inflammation. On histological preparations it is noticeable that the inflammatory infiltrate, as a rule, is localized around the glands. Against the background of focal mixed-cell inflammatory infiltration and congestion, the "thickening" of the secret, the formation of starchy little bodies - corpora amylacea is often noted. The staining of histological sections of the prostate by the corpora amylacea with Congo red confirms the amyloid nature of CA. CAs further contribute to the deepening of congestion, impeding the drainage of prostatic content.

In our previous study of intraluminal inclusions at BPH, it was found that prostatoliths were showed up in 27.6 ± 3.48 % among the histological specimens studied. Despite the frequent combination of the investigated features in patients with prostatolithiasis, no significant correlation was found between the presence of stones and CA in the prostate. A strong correlation was found between inflammation, stagnation and the formation of concrements, indicating the important etiopathogenetic role of these factors for prostatolithiasis [43]. These data allow us to better understand the mechanism of formation of prostatic calculi in the prostate.

According to electron diffraction (ED), infrared spectroscopy (IRS) and scanning electron microscopy with X-ray microanalysis (SEM / EDX), carbonate bioapatite with a high probability of minor inclusions has been found to be the mineral base of prostate concretions. PEM results show the morphological uniformity of crystalline particles of prostatic calculi and their size (tens of nanometers) is quite small. This is a testimony to their predominant formation by direct precipitation with calcium and phosphate supersaturated biological solutions than the origin and maturation on the surface

of biological tissues (in this case, corpora amylacea). Given the presence of carbonate substitutions according to the IRS, it is likely that pathologic calcifications of the prostate are not phase "pure" calcium apatite, but refer to apatites that have isovalent and heterovalent substitutions in the cationic and anionic lattices. However, the considerable concentration of zinc in the material of concrements does not allow to fully attribute it to the crystalline lattice of apatite (cationic substitution), but rather indicates a preferential stay in the non-apatite component of the deposit. In general, the presence of zinc is easily explained by its essentiality, tropism to the prostate, by the presence of coenzyme in many enzymes.

In our study, prostatic calculi with internal localization in the organ - prostatic glands or fibrotic tissue of the prostate - were selected for the material study. The concrements that could have come from the urinary system (prostatic part of the urethra) were not detected. This is explained by the fact that no prostatic calculi was found in the study, which consisted of oxalates, urates or other salts characteristic for the urinary system. The irregular rounded shape of the prostatoliths is due to their forced placement in the lumen of the glands of the prostate, and the stratification of the structure and the different size of the layers of mineral matter indicate the cyclical course of the pathological process with exacerbations and periods of remission. It seems that in the periods between the formation of the biomineral layers of the prostatoliths, proteins and microelements from the environment of the prostate enlightenment, which were "closed" between the hydroxyapatite plates of the concretions, were included in the concretions. It is this type of protein deposition that is detected during atomic force microscopy (AFM) and during the Western blot analysis.

Considering the results of pathomorphological examination and the data of physical material science, it can be concluded that the precipitating mechanism of concrement

formation in the prostate is the direct precipitation from the secretion of the prostate, and not the dystrophic calcification of starchy bodies.

The mineral basis of prostate concretions is carbonate bioapatite with little incorporation of foreign chemical elements.

Thus, the stability of the form and mineral composition of prostatoliths in all the tested samples testifies to the similarity of the mechanisms of formation of concrements and the regulation of the process of biomineralization in the prostate. The obtained results have allowed us to propose the following scheme of formation of a “circulus vitious” which arises at development of pathological biomineralization in a prostate (fig. 2.42)

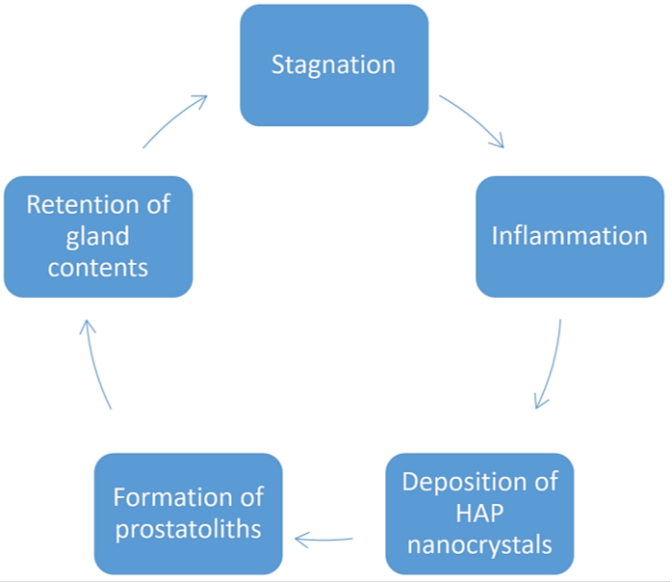

Figure 2.42 – The scheme of " circulus vitious" in prostatic lithiasis

2.4 Pathological biomineralization of the gallbladder

Among the diseases of the gallbladder, which accompanied by manifestations of pathological biomineralization, the most common are gallstone disease, which is manifested in the wall of the gallbladder with signs of chronic cholecystitis, carcinoma of the gallbladder with calcifications and the so-called porcelain gallbladder (PGB) [147]. Clinical importance of pathological biomineralization of the wall of the porcelain gallbladder is that it is associated with gallbladder cancer (GBC) in 12-62 % of cases [148, 149].

PGB is a rare manifestation of chronic gallbladder disease and is characterized by massive calcification throughout the wall and occurs in 0.06-0.8 % of cholecystectomies [150]. The causes of PGB are unknown. The porcelain gallbladder was first described in 1929 [149]. Pathology is more common in women than in men (5: 1 ratio). The age of most patients ranges from 50 to 70 years [151, 152]. Perhaps this reflects the overall statistics, because the disease of the GB, and directly gallstone disease are more common for women. PGB is believed to be a complication of chronic cholecystitis due to prolonged inflammation. This is confirmed by the fact that the prevalence of PGB increases with age and disease duration over 10 years [153].

There are two types of porcelain gallbladder, depending on the degree of calcification: full (occupies the entire organ, pierces the muscular layer) and incomplete (multifocal, dot deposits) [154].

Important is the issue of differential diagnosis of gallbladder adenocarcinoma with numerous dotted intracellular calcifications and incomplete type of porcelain gallbladder. It is believed that intracellular calcification of the GB is due to dystrophic calcification of the tumor necrosis foci. It is the reduction of metabolism and carbon dioxide that leads to a localized alkaline environment that causes mucin glycoproteins

to behave as ion exchange resins. This factor can cause the deposition of calcium compounds [155]. Most reports of calcified adenocarcinoma of the internal organs and gallbladder have been presented with a mucinous type of adenocarcinoma with multifocal, point calcifications, which are usually localized at sites of mucus accumulation.

Historically, perceptions of the relationship between PGB and the risk of cancer of the GB have changed. The first works of the 60–70's of the XX century. described a rather high incidence of malignancy (malignant degeneration) of PGB – up to 62 % [148, 153, 155]. Recent studies have shown that the incidence of carcinoma on the background of PGB ranges from 0 % to 5 % [154]. Some studies indicate that the full type of PGB is not associated with cancer of the GB [153].

The results of our study and the literature review data can be presented in Table 2.1 [156].

Table 2.1 – Pathohistological features of forms of gallbladder calcification

Indicator	**Chronic calculous cholecystitis**	**Gallbladder cancer with calcines**	**Porcelain gallbladder**
Size	Unchanged	Often increased	Reduced in size
Wall thickness	Slightly thickened	Thickened	Thickened
Microscopic description	In the wall of the GB can be distinguished layers of the usual structure, fibrotic changes, hyalinosis, intense mixed-cell inflammation of the mucous membrane	The gallbladder wall is fibrous, hyalinized with atypical glandular structures that grow invasively	The amount of cellular elements, massive deposits of small and large fragments of biominerals are sharply reduced in the wall of PGB
Localization of calcifications	In the cavity (in the form of stones), rarely in the walls as single foci	In the walls of the organ (in the tumor), it looks like a single focuses	The walls are totally calcified, a continuous layer, sometimes occupying the whole organ
The composition of minerals	calcium carbonate, dolomite	hydroxyapatite	hydroxyapatite
Fibrosis	moderately expressed	expressed	expressed
Hyalinosis	moderately expressed	expressed	expressed

Morphological and physicochemical analysis of mineral component of gallbladder tissue in its pathology

The study of the mineral component of the gallbladder tissue was carried out according to the scheme adapted to the situation: soft tissue staining with available biomineral component by alizarin red, scanning electron microscopy (SEM) of a section of mineralized tissue with X-ray microanalysis (SEM / EDX), X-ray diffraction (XRD) of mineralized fragments and atomic force microscopy (AFM) examination.

The study of the biomineral component of the pathological tissue of the GB was complicated by the small content of the GBC tissue, which could not be isolated in quantities sufficient for x-ray diffraction and other methods of applied material science. The determination of the crystalline phase for GBC was performed by examining tissue sections using SEM with microanalysis. On the other hand, the soft tissue of the wall of the GB with the effects of gallstone disease did not contain a mineral component. The study of the crystalline phase was carried out on calcium-containing concretions of the cavity of the GB by means of x-ray diffraction of their ashes.

Scanning electron microscopy of specimens of PGB wall showed massive deposits of biomineral matter in the tissue. These calcifications, according to EDX, consisted of calcium and phosphorus (Fig. 2.43) with a ratio of 1.4–1.6:1, which corresponds to the structure of hydroxyapatite [157].

In the study of the wall of the gallbladder with GBC using SEM revealed one foci of calcification with uneven edges, closer to rounded. Energy-dispersive x-ray analysis of the detected biomineral deposit showed a predominance of calcium and phosphorus with a ratio of 1.6: 1, which also corresponds to the structure of hydroxyapatite (Fig. 2.44).

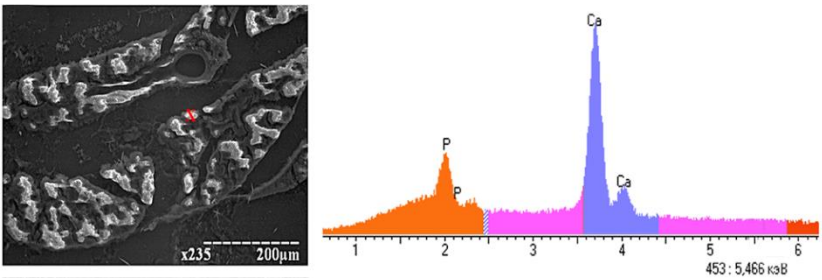

Figure 2.43 – Scanning electron microscopy with microanalysis. A. Slice of PGB tissue. The location of the microanalysis is indicated by a red label. X-ray diffraction pattern of the mineral component. The magnification and marker are indicated in the lower right corner of the microphoto

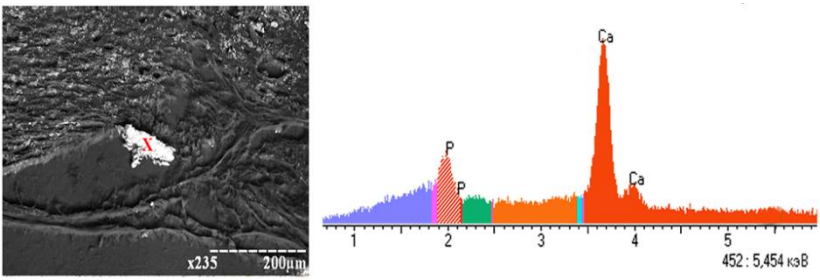

Figure 2.44 – Scanning electron microscopy with microanalysis. A. Slice of PGB tissue. The location of the microanalysis is indicated by a red label. X-ray diffraction pattern of the mineral component. The magnification and marker are indicated in the lower right corner of the microphoto

In the study of the biomineral component of PGB by means of X-ray diffraction of samples (Fig. 2.45), full compliance with the structural data of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, JCPDS No. 9-0432) was established.

Significant expansion and overlapping of diffraction peaks indicates a low degree of crystallinity of the material under study. The estimation of the crystallite size according to Scherrer [157] shows very close values to the typical size of bone crystallites. It is necessary to note some elongation of crystals of hydroxyapatite, which speaks of their similarity to lamellar or rod-like morphology characteristic of bone tissue or similar synthetic materials (Fig. 2.45).

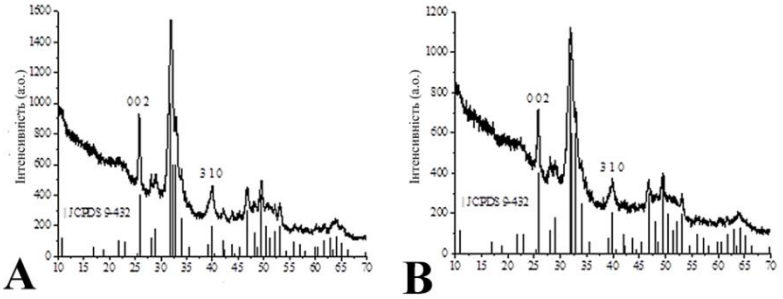

Figure 2.45 – X-ray diffraction patterns of biominerals of the gallbladder wall; the vertical lines correspond to the angular positions and relative intensities of the lines by JCPDS standard No. 9-0432

Structural studies of cavity concretions in gallstone disease of the gallbladder showed that the main crystalline phase of the biomineral deposit is calcium carbonate ($CaCO_3$, JCPDS No. 83-577 or/or JCPDS No. 88-1810), which was characterized by a high degree of crystallinity (Fig. 2.46).

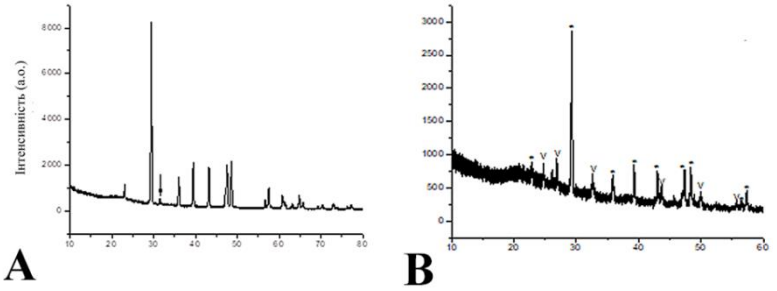

Figure 2.46 – X-ray diffraction patterns of pathological mineral formations inside the GB; the left arrow indicates the strongest line of additional calcium-phosphate phase (β- $(Ca, Mg)_3(PO_4)_2$ and/or apatite, right: * calcite line, JCPDS No. 88-1810, v – vaterite line, JCPDS No. 74-1867

In addition to calcium carbonate, in some samples other structural phases and forms of calcium carbonate, such as β-tricalcium magnesium phosphate $(Ca, Mg)_3(PO_4)_2$, dolomite, vaterite (JCPDS No. 74-1867), calcite (JCPDS) were also found in trace amounts (several percents). No. 88-1810).

The results of the study of crystalline phases of the gallbladder are shown in Table 2.2.

Atomic force microscopy of tissue sections was applied to study morphology and the three-dimensional properties of the biomineral component of the walls of the PGB and the GBC. For PGB, it was found that even at the nanoscale level, the fiber structure of biomineralized tissue remains, which also forms a mesh structure (Fig. 2.47 A). Another unexpected result of the study of the biominerals of PGB was the detection of extraordinary durable properties of this tissue - about 120 GPa, which is higher than many metals and synthetic materials. The high adhesiveness of this pathobiological material was also detected.

Table 2.2 – The results of the study of the phase composition of GB biominerls

Case	Age	Sex	Pathology	The crystalline phase of the mineral
1	58	F	PGB	HA
2	59	F	PGB	HA
3	60	F	PGB	HA
4	64	F	PGB	HA
5	66	F	PGB	HA
6	48	F	ChC	Calcite, TCMP
7	50	F	ChC	Calcite, TCMP
8	52	F	ChC	Calcite, vaterite
9	55	F	ChC	Calcite, vaterite
10	55	F	ChC	Calcite, dolomite
11	60	F	ChC	Calcite, dolomite
12	62	F	ChC	Calcite
13	63	F	ChC	Calcite, dolomite
14	48	F	ChC	Calcite, vaterite
15	51	F	ChC	Calcite
16	57	M	GBC	HAP
17	58	F	GBC	HAP
18	70	F	GBC	HAP
19	75	F	GBC	HAP
20	78	F	GBC	HAP

Notes. HAP – hydroxyapatite, TCMP – tricalcium magnesium phosphate

The biominerals that were found in the tissue of GBC were objects of amorphous structure, with rounded layers (Fig. 2.47 B). They had a rather high adhesiveness of the material, but the durability of the "cancer" biominerals was much lower (about 3 times - about 40 GPa).

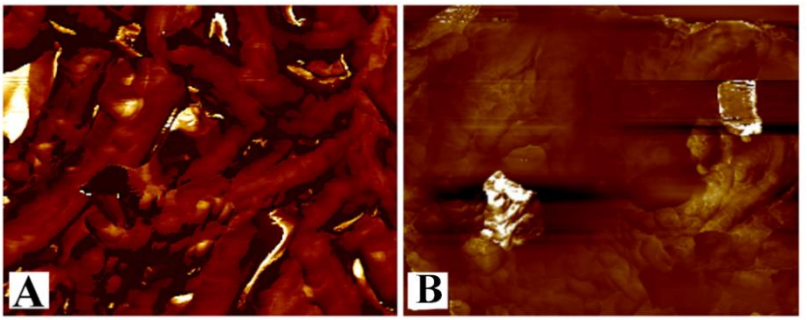

Figure 2.47 – Investigation of pathological gall bladder biominerals by atomic force microscopy. A. An image of a 5-nm area of the surface of the PGB biomineral. B. An image of a 5-nm area of the surface of the GBC biomineral

Of course, such results of AFM are encouraging, in terms of possible study of the development of a new type of biocomposite synthetic materials for industry and medicine. However, these results were obtained: 1) for the first time; 2) on a limited number of samples. Further studies with a sufficient number of samples for statistical processing are required to establish the fairness or inaccuracy of the results obtained and their reproduction.

Thus, different topology and conditions of formation of biominerals in the pathology of GB lead to the formation of different crystalline phases and/or different structural organization of the biominerals of the investigated organ. This indicates directly different mechanisms of development of pathological biomineralization. For example, "wall" biominerals are represented by hydroxyapatite and "cavity" by calcium carbonate with phosphate impurities. In addition, even among hydroxyapatites that form in the wall of the GB, different causes

and conditions of development lead to different structural and physical properties of biominerals.

A detailed study of the pathological biominerals of GB using additional methods of applied material science showed their different mineral composition. X-ray diffraction an alysis of the mineral deposits of the PGB wall revealed the presence of hydroxyapatite. Calcium-containing concretions of GB, according to the results of material research, consisted of calcium carbonate, in the presence of other trace amounts of calcium phosphate phase (vaterite, dolomite). The difference in the mineral composition of biominerals may be due to different conditions and mechanisms of their formation. Obviously, in the wall and cavity of the GB, there is a difference in the environment, pH, as well as the impact of various pathological processes. There is a large amount of sodium bicarbonate in bile as part of the powerful buffer system characteristic of this part of the gastrointestinal tract. It is obvious that it is the excess of bicarbonate ions that causes the formation of calcites in the cavity of the GB. One of the possible causes of pathological biomineralization in the gallbladder wall may be the lack of phosphorus in the internal environment of the gallbladder and the specificity of the conditions of formation of biominerals on the wall and in the cavity of the GB.

Paying attention to the peculiarities of the biological consequences and directionality of the processes of pathological biomineralization in the tissues of the GB, it should be noted that, among 5 clinical cases of PGB, no signs of malignant tumor growth or phenomena of any proliferative activity of the epithelium or other cells were detected. Therefore, based on the results of our own research and analysis of literature, we can conclude that the widespread (total) biomineralization of the GB has no clear connection with the emergence of malignant tumors. On the other hand, small foci of calcification in the wall

of the GB are a prognostic sign of possible association with GBC.

The different crystal-chemical characteristics of the mineral deposits of the wall of the PGB and the stones of the cavity of the GB indicate different conditions, causes and mechanisms of their formation. Different topology and formation conditions lead to the formation of different crystalline phases and/or different structural organization of the biominerals of the GB. This indicates directly the versatile mechanisms of development of pathological biomineralization. For example, "wall" biominerals are represented by hydroxyapatite and "cavity" by calcium carbonate with phosphate impurities. In addition, even among hydroxyapatites which form in the wall of the GB, polyetiology and developmental conditions lead to different structural and physical properties of biominerals.

We should highlight promising line of research of biomineralization processes of the fibers of the wall of the GB under the conditions of development of PGB. A deeper and numerical study of this problem may lead researchers to develop a new type of biocomposite synthetic materials for industry and medicine.

2.5 Pathological biomineralization in other organs

In all the investigated cases, the structural-phase and chemical analysis of pathological biominerals of the thyroid gland corresponded to calcite. It should be noted that calcite in the human body is formed only in the inner ear (otoliths, normal) and gallbladder (part of the stones, pathology). In other tissues, hydroxyapatite is predominantly formed [29, 31, 33, 37, 43, 49, 53]. The main reason for the formation of calcite in the tissues of the thyroid gland is the presence of a large number of bicarbonate ions there, which is the determining building material for pancreatitis [158].

Therefore, the study found that the presence of pancreatitis is accompanied by significant morphological remodeling of the thyroid gland. Histological examination of the thyroid gland revealed signs of chronic pancreatitis, fibrosis, atrophy and edema of the glandular tissue, systemic enlargement of the ducts of the gland, focal mixed-cell inflammatory infiltrates, plethora of blood vessels. The main crystalline phase of pancreatitis was calcium carbonate in the form of calcite.

Structural-phase and chemical composition of the eye biomineral was established using the methods of applied material science. The presence in the pathological formation of the apatite crystalline phase with relatively small crystallite sizes with a defective crystalline lattice is quite typical in many cases of ectopic biomineralization.

Thus, during the pathomorphological study of the biominerals of the eye, salivary, pancreas and mammary glands, some common features were identified. With the exception of pancreatitis, which is characterized by the presence of calcite, all the concretions were calcium-phosphate-type biomineralization, namely, they were represented by hydroxyapatites. For malignant ductal tumors of the salivary and mammary glands, a similar mechanism of mineral formation was observed, which was conditioned by ductal localization of tumors and the presence of a large amount of necrotic tissue in the parenchyma of tumors.

Since the biomineralization of soft tissues of living organisms is at the intersection of many scientific fields and areas, there are some difficulties in the systematization of the knowledge gained, due to a large amount of diverse information available. Traditional perceptions of biomineralization in soft tissues have been based on the predominance of general or local factors in the mechanisms of its development and the widespread perceptions of molecular and cellular interactions in the foci of occurrence of calcifications in previous decades. Thus,

metastatic, dystrophic and metabolic calcifications were distinguished. The widespread ectopic calcification arising on the basis of systemic disturbance of mineral metabolism was defined as metastatic calcification. It has been associated with the onset of hypercalcemia, which has evolved for a variety of reasons, and the subsequent fallout in the tissues of "excess" calcium compounds from biological fluids. If hypercalcemia was absent, ectopic biomineralization was defined as dystrophic calcification. It was the result of damage, disease, death or ageing of soft tissues. Dystrophic calcification has previously been thought to be characterized by the deposition of calcium compounds in tissues that are in a state of necrosis or deep dystrophy. The mechanism of dystrophic calcification was described as follows: destructive tissues are a source of phosphatases and alkaline environments. It should be noted that ossification, which is sometimes found in the same diseases, is also considered a manifestation of dystrophic calcification.

In cases where the explanation of the causes and mechanisms of biomineralization was difficult, the metabolic type of calcification was additionally highlighted. Thus, metabolic calcification was referred to as the so-called "lime gout", a disease of unknown etiology, which was associated with the instability of the body's buffer systems.

In recent years, many new mineralization factors have been explored and existing mechanisms of this process have been identified and refined. In numerous works on biominerology, an active focus and adjustability of biological mineralization processes have been established, and numerous factors of the cellular, molecular and metabolic environment of this process have been discovered.

Summarizing the results of our study of biomineralization of soft tissues under various causal and topographic conditions, we can distinguish the main points of pathological biomineral formation in human organisms.

According to the results of our research, pathological biomineralization develops by two main physical and chemical mechanisms: matrix deposition of biomineral deposits and direct deposition of biomineral compounds from a supersaturated solution. Matrix biomineralization can be divided into parenchymal and stromal, as it occurs in the presence of a surface for deposition. Such a surface can be easily found in most internal organs and vessels. The presence of a large number of stromal elements such as connective tissue, small vessel walls, nerve sheaths, and parenchyma elements such as cell membrane fragments, organelles, nuclei (apoptosis and necrosis effects) create all the necessary conditions for the development of biomineral formation. This way biominerals in the walls of the aorta, aortic valves, eyes, thyroid gland, lungs are formed.

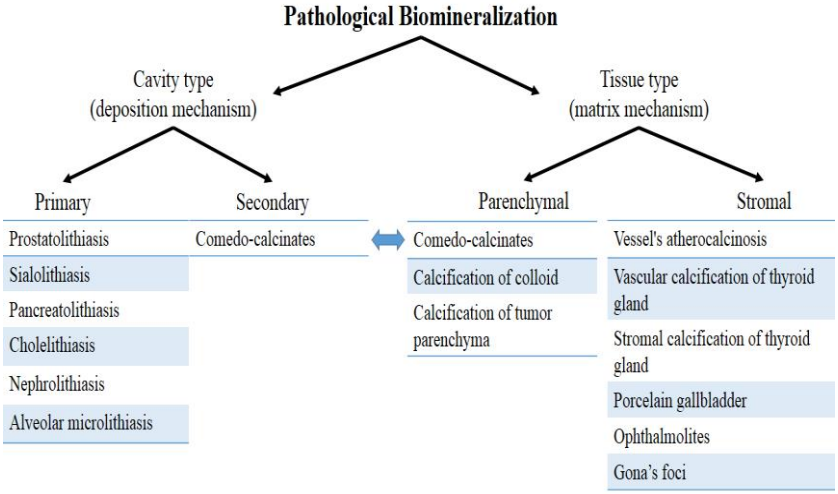

Figure 2.48 – Classification of pathological biomineralization of soft tissues by predominant physico-chemical mechanism and topography of biominerals

Formation of biominerals by a deposition mechanism is characteristic of "cavity" biomineralization. Favorable conditions for the formation of biominerals are present in biological cavities with limited or semi-open contours and filled with a biological fluid with a high content of "building material" for biomineralization. This mechanism is characteristic for the formation of prostatoliths, gallstones, pancrealiths and sialolites. Our studies also showed the presence of an intermediate variant of the development of the biomineralization process (Fig. 2.48).

This variant is characteristic for intraductal malignant tumors of the mammary and salivary glands. Intraductal gland cancer is characterized by the presence of comedo necrosis, which is actually formed in the lumen of the duct system. Over time, comedonecrosis undergoes biomineralization of the parenchyma by matrix type.

CONCLUSION

For over a billion years, biomineralization processes have provided living organisms with the conditions for locomotion, support, protection against predators, and even act as weapons. The formation of biominerals has undergone changes along with the evolutionary process. Of course, like any useful compensatory-adaptive reaction, biomineralization has a pathological reflection: many diseases are complicated by its excessive or untimely manifestations.

The study of biomineralization should aim to study the significance of this process for conditions of physiological norm and pathology. Only after that, it is necessary to develop ways to prevent, block or stimulate them. This is because in many cases the development of biominerals is a protective reaction of the body to damage by a pathogenic factor, so inhibition of biomineralization in some cases (complicated atherosclerosis, papillary thyroid cancer, osteoblastic bone metastases) can cause new pathological processes or complications of the disease in the body.

In most cases, the development of PBM causes significant harm to the body, significantly reducing the quality and life expectancy of patients. For such pathological processes it is necessary to study in detail the mechanisms of mineral formation in living tissues and to develop ways of preventing and blocking the development of "pure" pathological biomineralization.

A detailed study of the processes of biomineralization of soft tissues will allow us to take a fresh look at many established ideas about this process. New knowledge gained during the research stimulates the development of many promising areas in medicine, molecular biology and biotechnology of new materials. Thus, the presence of high levels of expression of certain proteins, and especially fragments of their micro-RNA

and DNA, will make it possible to diagnose and clarify the stage of major general pathological processes (malignant tumor growth, atherosclerosis) even before the appearance of widespread clinical manifestations. On the other hand, detection of the fact of directed elimination of intratumoral vessels by means of biomineralization processes will allow to start searching for targeted medicines for antitumor chemotherapy. A separate line should be allocated to the possibilities of research, isolation from the biological environment and synthesis of new promising biocomposite materials. Biological prototypes of this kind of materials occur at different stages of the development of biomineralization processes. For example, mineralized fibers of the wall of the porcelain gallbladder have extraordinary mechanical properties (durability, adhesion, hardness). Mineralized aortic tissue and valves show interesting data.

The prospect of searching for new biomarkers, chemical products, biocomposite materials will allow to reveal at the present level the relevance and further development of studies of biomineralization processes in living organisms.

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