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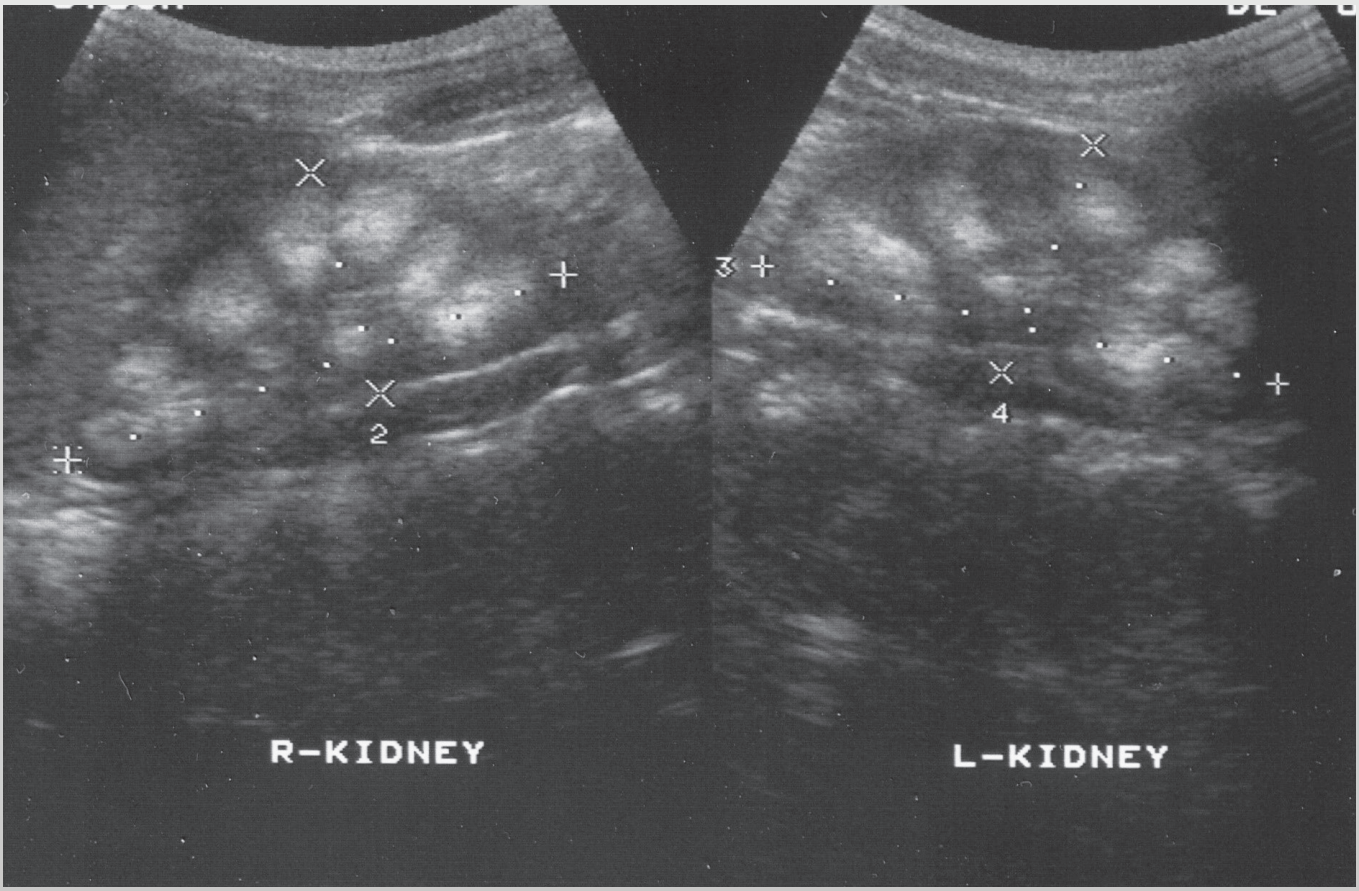

Bilateral medullary nephrocalcinosis in a baby with idiopathic infantile hypercalcemia.
(Taken from the paper by Velibor Tasic and Zoran Gucev)

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The cover image taken from the paper from this issue by Velibor Tasic, Zoran Gucev, "Vitamin D Supplements – Benefits and Risks".

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EDITORIAL

Dear Readers,

New issue of Facta is in front of you. We have a great pleasure to welcome the invited article authored by one of the most cited and famous European Pediatric Team from Macedonia, Prof dr Velibor Tasic, pediatric nephrologist and Prof. dr Zoran Gucev pediatric endocrinologist.

Due to its pleiotropic effects, "the sunshine vitamin D" showed many beneficial effects and it is widely used as panacea. Nowadays, many of these attributed effects are challenged. Tasic and Gucev provided a review of the most recent data of *pro et contra* facts for such use of vitamin D. Showing that some individuals with variant of CYP24A1 gene are at an unacceptable risk of developing severe, life threatening complications such as infantile hypercalcaemia, (previously called idiopathic), they warned us to respect interindividual differences in vitamin D response. In Macedonia, seven babies were diagnosed on clinical basis with idiopathic infantile hypercalcaemia. Testing on *CYP24A1* mutations revealed that all had typical Central European E143del mutation. In adults this abnormal vitamin D degradation pathway is responsible for nephrolithiasis, nephrocalcinosis, hypercalciuria, intermittent episodes of hypercalcemia. In the absence of hypercalcemia suppressed PTH may be a clue to proper diagnosis in these individuals.

The authors made important conclusions and future directions for exploring the prevalence of vitamin D metabolic defect. This is also very important for prenatal or early postnatal diagnosis of *CYP24A1* mutation carriers for implementation of early preventive measures.

Editor-in-Chief
Ljiljana Šaranac

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Invited Review Article

VITAMIN D SUPPLEMENTS – BENEFITS AND RISKS

Velibor Tasic, Zoran Gucev

University Children's Hospital, Medical School, Skopje, Macedonia

Abstract. *Vitamin D has several important functions including absorption of calcium and phosphorous, and facilitating normal immune system function. Sufficient amount of the vitamin is required for normal growth and development of bones and teeth, as well as improved resistance against certain diseases. There is growing evidence that there are huge benefits of vitamin D in promoting the human health, not only in infants for prevention of rickets but also effects on the immune system, blood pressure, reducing the risk of some cancers, prevention of diabetes mellitus type 1 trough stimulation of the pancreatic beta cells to secrete insulin. In contrast to these benefits certain patients genetically predisposed are at risk to develop a serious even fatal disease such as idiopathic infantile hypercalcemia. Withdrawal of vitamin D and reduction of calcium intake are lifesaving interventions for these babies. Recently it was found that recessive mutations in CYP24A1 gene are responsible for this disease. This gene encodes the enzyme 24 vitamin D hydroxylase which is important in the degradation metabolic pathway of the vitamin D. Although it was generally believed that idiopathic infantile hypercalcemia is the disease limited to infancy a number of studies yields that adults may have serious morbidity including nephrolithiasis, nephrocalcinosis, intermittent episodes of hypercalcemia leading to chronic kidney disease and in few cases to end stage renal disease. Therefore one should be very cautious in liberal prescribing vitamin D supplements and excessive exposure to sunlight, particularly in individuals with genetic predisposition*.

Key words: *vitamin D, supplements, CYP24A1, toxicity, children, nephrolithiasis, nephrocalcinosis*.

General

Vitamin D is called the "sunshine vitamin" because it's produced in the skin in response to sunlight. Vitamin D is a fat-soluble vitamin in a family of compounds that includes vitamins D1, D2, and D3. Vitamin D has several important functions including absorption of calcium and phosphorous, and facilitating normal immune system function. Sufficient amount of the vitamin is required for normal growth and development of bones and teeth, as well as improved resistance against certain diseases. The deficiency of vitamin D increases the risk of developing bone abnormalities such as osteomalacia or osteoporosis. It is believed that a 10 minutes a day of mid-day sun exposure is sufficient for production of adequate amount of vitamin D. Besides getting vitamin D through sunlight, it is provided through intake of certain foods and supplements [1]. Certain environmental factors and lifestyle influence the ability to get sufficient amounts of this vitamin through the sun alone such as pollution, use of sunscreen, spending more time indoors, long working hours in offices, living in big cities where buildings block sunlight. Therefore it is important to provide additional amounts of vitamin D from sources other than sunlight exposure. The recommended daily doses of vitamin D according to the Institute of Food and Agricultural Sciences (IFAS) [2] are:

- children and teens: 600 IU
- adults up to age 70: 600 IU
- adults over age 70: 800 IU
- pregnant or breastfeeding women: 600 IU

The consensus of scientific understanding appears to be that vitamin D deficiency is reached for serum 25-hydroxyvitamin D (25OHD) levels less than 20 ng/mL (50 nmol/L), insufficiency in the range from 20–32 ng/mL, and sufficiency in the range from 33–80 ng/mL, with normal in sunny countries 54–90 ng/mL, and excess greater than 100 ng/mL.

Health Benefits

There is growing evidence that there are huge benefits of vitamin D in promoting the human health, not only in infants for prevention of rickets but also effects on the immune system, blood pressure, prevention of diabetes mellitus type 1 trough stimulation of the pancreatic beta cells to secrete insulin.

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Malignancies

Vitamin D has strong anticancerogenic effect for development of malignancies of the breast, colon, prostate, ovaries, esophagus, and lymphatic system. Several studies have shown that increased dietary intake of vitamin D as well as higher blood levels of vitamin D are associated with a reduced risk of colorectal cancer [3,4,5,6]. In experimental studies it has been found that vitamin D prevents the development of cancer trough enhancement of cellular differentiation, decreasing cancer cell growth, stimulating apoptosis and reducing tumor blood supply and angiogenesis [7,8,9,10]. Randomized The Women's Health Initiative study did not confirm the beneficial effect of vitamin D supplements for an average period of 7 years in reducing the incidence of colorectal cancer [11].

The limitation of majority studies which deal with the beneficial effects of vitamin D to human health arises from the fact that in dietary studies vitamin D produced in the skin from sunlight exposure is not taken in consideration. In most studies vitamin D level is measured in the blood at a single point in time and this may not correspond to a person's true vitamin D status. One may speculate that people with higher vitamin D intakes or blood levels have healthier behavior in general which reduces the cancer risk.

Upper respiratory tract infections

The beneficial effects of the vitamin D were questioned in the VIDARIS study reported in JAMA in 2012 [12]. In this randomized, double-blind, placebo-controlled trial adult participants were randomly assigned to receive an initial dose of 200,000 IU oral vitamin D3, then 200,000 IU 1 month later, then 100,000 IU monthly (n = 161) or placebo (n=161) for a total for 18 months,. The endpoints of this study were the number of upper respiratory tract infection episodes, their severity, duration and days off missed work. The results of this study were disappointing; no statistical significant difference was found in none of tested parameters.

Hypertension

In a meta-analysis performed by Kunutsor et al. [13] including a total of 283,537 participants, the investigators found that for each 10 ng/ml increase in someone's vitamin D levels, there was a 12% lower risk of developing hypertension. Also the people with the highest vitamin D levels had a 30% lower risk of developing hypertension compared to the people with the lowest levels. The limitation of this meta-analysis is that the analyzed studies were performed in United States and one may wonder if these results could be validated in other populations.

In another American study researchers found that that for every increase in vitamin D supplementation and vitamin D levels in the body, systolic blood pressure decreased but there was no changes in the diastolic blood pressure [14].

The researchers of the Women's Health Initiative Randomized Trial assigned women to either receive 1,000 mg per day of calcium plus 400 IU per day of vitamin D or a placebo pill. The results showed that there was no difference in blood pressure changes between the groups [15].

The study from Denmark investigated the effect of vitamin D supplements on lowering blood pressure in people with hypertension [16]. The study period was 20 weeks and the subjects were randomized to take 3,000 IU vitamin D per day D or placebo. This study showed that subjects in vitamin D group lowered their blood pressure more than those in the placebo group. The second conclusion was that subjects in the vitamin D group who had low levels of vitamin D at the beginning of the study had a bigger reduction in their blood pressures.

The limitation of abovementioned studies is that the hypertensive subjects were taking their medication during the study period, so it is uncertain if the lowering of the blood pressure was due to vitamin D or prescribed antihypertensive therapy.

Diabetes

There is evidence from experimental studies that vitamin D treatment improves glucose tolerance and insulin resistance and that supplementation with vitamin D restores insulin secretion in animals [17]. This is an indirect effect which is mediated by the flux of calcium trough the cell membranes; therefore low levels of extracellular calcium diminish insulin secretion. There are epidemiological studies which revealed greater incidence of type 1 diabetes related to geographic variation. The study from Finland analyzed 10,821 children who were supplemented with different vitamin D doses [18]. An important finding from this study was that children who took 2,000 IU of vitamin D daily had 80% lower risk to develop type 1 diabetes. Another point from this study was that vitamin D supplementation during the first year of life was critical for development of type 1 diabetes.

The evidence supports that maintaining adequate vitamin D status during pregnancy, nursing, infancy, and childhood may help prevent type 1 diabetes [19]. It is still the matter of controversy weather genetics of type 1 diabetes place individuals at risk for vitamin D deficiency or vice versa vitamin D deficiency increases the risk for type 1 diabetes. There are no studies to show the beneficial effect of vitamin D on the treatment of type 1 diabetes after diagnosis. Several studies have examined the impact of vitamin D supplementation on reversing type 1diabetes, and they have not been successful [17].

Risks

Cardiovascular risks

There is evidence that vitamin D deficiency is associated with cardiovascular morbidity and mortality, but also there is some evidence that high levels of vitamin D may also be associated with adverse arterial remodeling and poor outcomes [20,21]. It has long been known from case series that vitamin D excess can lead to atherosclerosis

and vascular calcification in humans. In NHANES III study there was a U-shaped relationship between vitamin D and mortality risk, particularly in women, with 25(OH)D levels >50 ng/L [22]. Although 1 meta-analysis that included 8 studies that assessed relatively high (>65 nmol/L) levels of 25(OH) found no significant change in risk of cardiovascular disease, another meta-analysis reported evidence of increased mortality with 25(OH)D concentrations >97.5 nmol/L [23].

Amer and Qayyum found that excessive vitamin D levels above 21 nanograms per milliliter were associated with an increase in CRP, which is known inflammatory marker and which is associated with the stiffening of blood vessels and a greater risk of developing cardiovascular problems [24].

One may have in mind that the role of vitamin D in the prevention and management of cardiovascular disease as well as the dose-response relationship of potentially harmful effects still remain to be established.

Idiopathic infantile hypercalcemia

There is pediatric entity entitled idiopathic infantile hypercalcemia (IIH) which presents in infants who may be severely ill with vomiting, poor appetite failure to thrive, seizures and if unrecognized and inappropriately treated may die. Biochemically these babies have hypercalcemia, hypercalciuria and suppressed parathormon. Imaging studies reveal bilateral nephrocalcinosis (Fig 1). Withdrawal of vitamin D and reduction of calcium intake are lifesaving interventions for these babies. The etiology was unknown until 2001 when Schlingmann and the group from Munster reported in The New England Journal of Medicine that homozygous *CYP24A1* mutations were cause for this disease in majority of babies [25]. This gene controls the enzyme 24hydroxylase which function is to degrade vitamin D and prevent sufficient synthesis of calcitriol. The authors wanted to validate their findings and therefore tested adult patients from former East Germany who had had signs of vitamin D toxicity as infants. The practice in East Germany was to administer parenterally 2 million units of vitamin D during the first 2 years of life. Indeed these adults carried homozygous mutations in *CYP24A1*.

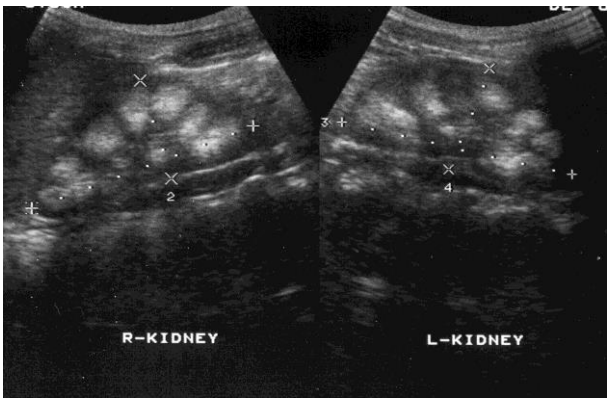

Fig. 1 Bilateral medullary nephrocalcinosis in a baby with idiopathic infantile hypercalcemia.

In Macedonia we have diagnosed on clinical basis 7 babies with IIH. We tested them for *CYP24A1* mutations and found that all had typical Central European E143del mutation. After the report in The New England Journal of Medicine there were additional reports in which *CYP24A1* mutations were found in adult subject with idiopathic calcium oxalate nephrolithiasis or unexplained nephrocalcinosis [26–33]. A study from Israel reported a small series of patients with nephrolithiasis/ nephrocalcinosis, even some of them progressed to terminal renal failure [33]. The etiology has not been established for decades and finally all were tested and found to carry *CYP24A1* mutations.

Recently in collaboration with Boston Children's Hospital (Harvard Medical School) using targeted next generations sequencing we diagnosed IIH in 12 year old girl who had incidental nephrocalcinosis [34]. She had normal growth and had not any problems as an infant. Along with this case and other study reports it is now clear that IIH is not the disease exclusively limited to infancy. This is important for these patients since they have to avoid lifelong vitamin D supplements and sunlight exposure. So it s questionable if IIH is a disease limited of infancy. The growing number of reports point that adult homozygous carriers of *CYP24A1* mutations may have serious morbidity – calcium oxalate nephrolithiasis, nephrocalcinosis, hypercalciuria, intermittent episodes of hypercalcemia. In the absence of hypercalcemia suppressed PTH may be clue to proper diagnosis.

Conclusion and Future Directions

Surely that vitamin D is very attractive for promotion overall human health. But one may have in mind that liberal administration of vitamin D supplements may have adverse effects in genetically susceptible individuals. Do we diagnose all patients with IIH? Is this only the tip of the iceberg? It seems that only patients with severe symptoms come to our medical attention. What can we do on the population basis? What is the prevalence of *CYP24A1* mutations in the Balkan populations? These questions remain to be answered in the near future. We can easily test for E143del. Family relatives will have great benefit of such testing. This is also very important for prenatal or early postnatal diagnosis of *CYP24A1* mutations carriers to implement early preventive measures.

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Review Article

INTERACTIONS BETWEEN SKELETAL SYSTEM AND MACROPHAGES IN HOMEOSTASIS AND BONE INJURY

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Abstract. *New insights about close connection between skeletal and immune systems have expanded vistas of modern medicine and tissue engineering. Intensive progress of osteoimmunology enabled the understanding of processes related to bone tissue from a completely new angle, both in physiological and pathological conditions. In this respect, macrophages stand out as cells which affect bone through the ability to secrete a range of different cytokines. Macrophages' activation is directed by environmental conditions which determine the phenotype and function of these cells. Acquired phenotypic and functional characteristics of macrophages are changed according to changes in their environment. Thanks to these abilities, macrophages have great impact on bone development, bone homeostasis and osteoreparatory process. During bone development, macrophages can affect osteoblast differentiation and matrix mineralization. Coordinated action of osteoclasts and osteoblasts is important in bone tissue remodeling process. Also, during osteoreparation macrophages are among the first cells that will come to the site of bone injury. Their impact on bone is particularly visible during inflammatory phase of fracture healing. Better understanding of mechanisms by which macrophages exert their influence on bone would be an important step in approach to more specific therapies that would modulate activity of these cells and might accelerate healing of bone defects.*

Key words: *macrophages, bone, bone homeostasis, osteogenesis, fracture, osteoreparation*.

Introduction

The belief that bones represent inert structures has been disproved long ago by abundant evidence that bone tissue is very dynamic and that it is in constant process of resorption and formation [1, 2]. There are numerous data on direct correlation between skeletal and immune systems. Among various cells of immune system, macrophages are those that stand out by their secretory products which directly affect osteogenesis and osteoreparation [3–5]. In addition, macrophages are very plastic cells since they adjust their activity and change their phenotype according to general state of the environment. They are involved in several stages of osteoreparation, and are especially important actors during initiation of bone tissue healing [4]. Therefore, the possibility of modulating macrophages' activity would be a useful tool in an attempt to control osteogenesis and osteoreparation, especially after bone tissue injury or in pathological conditions.

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Macrophages' Differentiation and Activation

Macrophages belong to a group of professional phagocytes which perform their functions thanks to numerous surface receptors and secretory products [6]. Almost all organs of the body contain tissue-resident macrophages, which play an important role in homeostatic processes [7, 8]. Macrophages have a wide range of morphological characteristics that correspond to their functional state and environmental conditions. Different subpopulations of tissue-resident macrophages exist in various tissues [8–10]. Depending on the tissue they are placed, tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), microglial cells (CNS), histiocytes (connective tissue), Kupffer cells (liver), and Langerhans cells (skin) [11].

The process of macrophages' differentiation should be distinguished from activation process, which means that differentiated macrophages through further stimulation increase their capability to exert certain functions. Tissue-resident macrophages are quiescent and characterized by low oxygen consumption, low expression level of major histocompatibility complex class II gene (MHC II), a little cytokine production and by preserved proliferative capacity. It is believed that there are two levels of macrophages' activation. Initial activation (priming) leads to increased expression of the MHC II gene, increased production of cytokines and reduced proliferative capacity.

Priming is usually achieved by low concentrations of interferons (IFNs) and it is a process of preparing for a quick response and reaction to other cytokines, although macrophages are not still fully activated [12]. Macrophages then react to secondary signals (e.g. tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS)) and become fully activated, which means that they lose their ability to proliferate, but gain ability to kill parasite and tumor cells, and all this is accompanied by increase in oxygen consumption, cytokines, reactive oxygen species (ROS) and nitric oxide (NO) secretion [13, 14].

Macrophages' Classification according to their Functional Characteristics

Macrophages show a remarkable plasticity through the ability to adapt their phenotype and function to environmental changes. Tissue injury, infections or tissue reaction to a foreign body excite quick response of these cells. Macrophage classification arises from their functional characteristics, surface markers and type of produced cytokines. According to their functional characteristics macrophages are usually classified as M1 or M2, i.e. classically and alternatively activated macrophages. This nomenclature is based on the type of T cells (Th1 or Th2) which influence macrophages' activation by distinct cytokines [15].

M1, i.e. classically activated macrophages, are referred as inflammatory and can be activated by IFN-γ, TNF-α and LPS. They are involved in defending the host against various pathogens and tumors. Macrophages of M1 type produce ROS and NO, high level of interleukin-12 (IL-12) and low level of IL-10 and also produce numerous pro-inflammatory cytokines including TNF-α, IL-1 and IL-6 [6].

M2, i.e. alternatively activated macrophages, are referred as anti-inflammatory according to their anti-inflammatory function, but they also regulate wound healing [6]. Within this type of functional macrophages there are three subtypes of cells with different physiological roles. M2a macrophages are involved in later events of tissue repair, and they are activated by cytokines IL-4 and IL-13. M2c macrophage subtype is induced by IL-10 or glucocorticoids, and this subtype has anti-inflammatory function. M2b macrophages also achieve anti-inflammatory activity via IL-10, but also synthesize pro-inflammatory cytokines (IL-1 and TNF-α), like M1 type macrophages [16].

The relationship between Skeletal and Immune Systems from Macrophages' Perspective

At the beginning of the new millennium osteoimmunology was defined as new branch of science that deals with interactions between cells of immune system and bone tissue cells [17]. Immune cells produce cytokines which can have a part in normal bone tissue healing [4], but also can affect appearance and flow of different pathological conditions [18].

The connection between bone and immune system exists on at least three levels. Firstly, bone marrow is anatomically located in bones, so the mutual interaction of immune and bone cells is unavoidable. Secondly, cells of immune system originate from hematopoietic stem cells of bone marrow, similar to osteoclasts which structurally and functionally belong to bone tissue. Thirdly, the two systems share various cytokines, growth factors, signaling molecules and transcription factors [19].

Connection and conditionality between cells of bone and immune system is clearly represented trough osteoclastogenesis, since many factors that affect precursors of osteoclasts can be synthesized by inflammatory cells too. Furthermore, osteoclasts and immune cells share the same progenitors through differentiation process [20]. Osteoclasts originate from bone marrow pluripotent hematopoietic stem cell and are by themselves specialized bone tissue macrophages [11, 21]. Likewise, individual macrophages can fuse together to form osteoclasts [22]. The two most important cytokines that are necessary for unobstructed osteoclastogenesis are receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which can be secreted among others by inflammatory cells. RANKL is a cytokine expressed by osteoblasts, stromal cells, and activated T lymphocytes [23] and belongs to TNF superfamily. RANKL binds to RANK-receptor which exists on the surfaces of osteoclast precursors. Osteoprotegerin (OPG), secretory product of osteoblasts and numerous hematopoietic cells, is RANKL-competitor and has anti-osteoclastogenic function [24]. M-CSF is produced by bone marrow stromal cells, osteoblasts and T lymphocytes and it is responsible for proliferation and survival of osteoclast progenitors, as well as mature osteoclasts [25]. The fact that these two factors can be synthesized by cells of immune system indicates that in this way immune system can affect bone tissue. This correlation is particularly visible in some bone diseases [26].

Macrophages/monocytes can regulate bone development and homeostasis through secretion of numerous cytokines and other molecules, although their role in abovementioned processes is still not fully understood. Many of these secretory products are pro-angiogenic and pro-osteogenic [27]. It has been experimentally proved that macrophages are involved in osteoblast differentiation [3] and mineralization process [3, 28]. In addition, macrophages may activate other cells from their environment to secrete certain cytokines important for the osteogenic process [27]. Chang and coworkers point to macrophage population termed OsteoMacs in murine and human osteal tissue, significant in bone homeostasis. OsteoMacs are defined as stellate-shaped resident bone tissue macrophages located on endosteal and periosteal surfaces. Difference between OsteoMacs and osteoclasts is, among others, based on $F4/80^{+}TRAP^{-}$ phenotype of OsteoMacs and $F4/80^{-}$ $TRAP^{+}$ phenotype of osteoclasts. Also, OsteoMacs in

physiological conditions are not osteoclast precursors, while they may be in pathological conditions. These cells interact with osteoblasts, regulate their function and mineralization process through induction of gene for osteocalcin *in vitro*. [29]. OsteoMacs at bone modeling and remodeling sites form canopy structures over mature osteoblasts. Depletion of these cells leads to disappearance of mature osteoblasts from bone modeling surfaces. During bone remodeling OsteoMacs, like osteoclasts, provide coupling signals, most probably transforming growth factor beta (TGF-β) and ephrin B2 to osteoblasts, affecting bone formation [30].

Another unique ability of macrophages is to quickly respond to chemoattractants from the site of tissue injury. During fracture healing, macrophages come to the site of injury and release various cytokines that promote angiogenesis and recruitment of mesenchymal stem cells [27]. Presence of blood vessels and mesenchymal stem cells at defect site is crucial for proper osteoreparatory process [31, 32]. All of these macrophages' capabilities are in favor of their potential use in bone tissue engineering.

Inflammatory process plays an important role in initiating bone regeneration after injury. On the other hand, some inflammatory diseases or reactions to implanted material can lead to chronic inflammation, which has a destructive effect on bone tissue [33]. One such example of bone destruction associated with inflammation is reumatoid arthritis [34]. Therefore, studies concerning control of inflammatory signal are of the great significance.

The role of macrophages in the process of fracture healing is discussed in the following sections.

Repair of Bone Defects

Bone healing process usually goes through three dynamic phases that overlap each other and are named inflammatory, reparative and remodeling phase. Therefore, repair of bone defects (fractures) is characterized by an initial inflammatory reaction accompanied by cell proliferation and remodeling, which ultimately leads to bone reconstruction. The main actors of inflammatory process are macrophages, which migrate to the site of injury [4]. These cells also release factors involved in the formation and resorption of bone tissue.

Inflammatory phase

Together with bone damage, as consequence of fracture, damage of surrounding tissues and blood vessels also develops. Blood coagulation results in formation of hematoma. Due to blood vessels injury in the zone of bone fracture, lack of oxygen and nutrients occurs, leading to premature cell apoptosis and to the formation of necrotic tissue. Necrotic tissue, platelet-derived growth factor (PDGF) from blood clot and growth factors from extracellular matrix (TGF-β for example) act as chemoattractants for inflammatory cells (macrophages, monocytes, lymphocytes and neutrophils) and fibroblasts, and provoke acute inflammatory response. This initial phase of bone tissue healing reaches its maximum 24-48 h after injury and completes in about 1 to 2 weeks [35, 36]. Actually, it is believed that these first 2 weeks are the milestone in bone healing process [37].

Inflammatory phase is characterized by dynamic processes such as formation of granulomatous tissue, ingrowth of blood vessels and migration of mesenchymal stem cells to the fracture site [38, 39]. Likewise, levels of several pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-11 and IL-18 are significantly increased [36, 38]. Although it is known that extended or chronic expression of pro-inflammatory cytokines might have negative effect on bone, short-term and highly specific secretion of these molecules is extremely important for tissue regeneration [40, 41]. These signals recruit inflammatory cells and promote angiogenesis [38]. It is believed that TNF-α as a product of inflammatory cells, especially macrophages, mediates the induction of secondary pro-inflammatory signals, which are chemoattractants for different cells and also can induce osteogenic differentiation of osteoblast-like cells [42, 43, 44]. Along with them, TGF-β1 and PDGF from blood clot also serve as guides to differentiation and proliferation of mesenchymal stem cells [45]. Over time, the acute inflammatory response is being replaced by the next phase.

Reparative phase

Reparatory phase starts with reorganization of hematoma. Numerous cells which came to the fracture site during inflammatory phase produce callus. Callus consists of cartilage and immature bone tissue and has function to increase stability of the fracture. Formed cartilage through ossification process becomes bone, under the influence of TGF-β2, TGF-β3, bone morphogenetic proteins (BMPs) and other signaling molecules [35, 38, 42, 46]. During reparative phase inflammatory cells and pro-inflammatory cytokines are absent [39].

Remodeling phase

During remodeling phase through the activity of osteoblasts and osteoclasts initial immature woven bone is replaced by mature lamellar bone. This phase, which begins 8 to 12 weeks after injury, is strongly osteoclast-dependent and it is regulated by a number of pro-inflammatory signals like IL-1, IL-6, IL-11, IL-12 and TNF-α [36, 38, 39]. Remodeling phase is the longest phase during bone healing process and can last up to several years.

The role of Macrophages during Fracture Healing Process

Macrophages play a significant role in bone healing process, in initial as well as the final stage. Immediately after fracture, macrophages along with neutrophils and lymphocytes penetrate into hematoma. Monocytes/

macrophages produce BMP-2, one of the key factors involved in the early osteogenic process. In fact, BMP-2 directs stem cells toward osteoblast differentiation *in vitro*, as well as *in vivo*. Pirraco and coworkers used experiments with co-cultures of human peripheral blood monocytes/macrophages and human bone marrow stromal cells (hBMSCs) which have shown that hBMSCs from co-cultures have higher proliferative capacity and higher alkaline phosphatase activity in regard to hBMSCs monocultures [47]. Schlundt and colleagues have worked with murine experimental model which included macrophage reduction using clodronate liposomes during bone healing process. In their experiments macrophages' reduction had no effect on early stages of fracture healing, while they had altered endochondral ossification through delayed hard callus formation [48].

Bone tissue is well vascularized so angiogenesis and vascularization are essential for unobstructed repair of bone tissue after injury [31, 49, 50]. According to literature data it is known that macrophages are able to affect all stages of angiogenesis thanks to their secretory products [51]. Stimulated macrophages release pro-angiogenic cytokines and growth factors, as well as enzymes that degrade extracellular matrix and enable releasing of "trapped" growth factors (bFGF, TGF-beta, GM-CSF) which also have proangiogenic activity [52]. Inclusion of macrophages (induced from THP-1 monocytic cell line treated with PMA (phorbol-12-myristate-13- acetate)) in co-culture made of human outgrowth endothelial cells (OECs) and primary osteoblasts leads to multiplying of microvessel-like structures formed by OECs and higher production of vascular endothelial growth factor (VEGF) compared to co-culture. Likewise, in triple-culture expression of IL-6, IL-8 and TNF-α was upregulated, indicating beneficial effects of pro-inflammatory cytokines in osteoreparation [53].

M1 type macrophages are the first that could be found at the site of tissue injury, with role to engulf necrotic material and to synthesize pro-inflammatory cytokines, ROS and NO. Guihard and coworkers found that M1 type macrophages stimulate osteogenic process through production of Oncostatin M (OSM), member of IL-6 cytokine family, which induce osteoblast differentiation and mineralization. [3]. Other experiments based on juxtacrine interaction in co-cultures composed of primary mouse macrophages and bone marrow stromal cells (BMSCs) resulted in enhanced proliferation and migration of stem cells which was mediated with increased macrophage IL-6 production in these co-cultures [54].

M1 macrophages are later replaced by M2 type that produces IL-10, TGF-β, as well as other anti-inflammatory cytokines, which are essential for proper wound healing. Actually, due to their plasticity macrophages can switch from M1 to M2 phenotype [55, 56]. It has been experimentally proved in mouse osteotomy model that induction of M2 macrophages during fracture healing process enhances bone formation [48]. Loi and colleagues had investigated the effect of M1 and M2 type macrophages on osteogenesis *in vitro* in co-cultures of polarized primary murine macrophages and preosteoblastic MC3T3-E1 cells [57]. In each co-culture type osteogenic differentiation of MC3T3-E1 cells was increased and switching of macrophage phenotype from M1 to M2 through IL-4 application had enhanced osteogenic ability of MC3T3-E1 cells in co-cultures. It has been confirmed by these experiments that inflammatory phase is necessary before healing process is initiated.

Above all, action of these two types of macrophages had to be balanced. If M1 type macrophage activity overcomes macrophages of M2 type, that can lead to further tissue damaging, while the opposite case can lead to fibrosis [58].

During inflammatory phase macrophages remove necrotic tissue and secrete a number of pro-inflammatory cytokines such as TNF-α and IL-1. The aforementioned pro-inflammatory cytokines reach maximal concentration 24 h after tissue injury [59]. At fracture site TNF-α can have a dual function that depends on which of the two cell receptor (TNFR1 and TNFR2) TNF-α binds [36]. IL-1 can exists in two forms: IL-1α and IL-1β. While IL-1α upregulates inflammation [60], IL-1β is thought to have a positive effect on mesenchymal stem cells differentiation into osteoblasts [61] and proliferation of osteoblast-like cells [43].

During remodeling phase TNF-α concentration rises again [59]. This cytokine binds to TNFR1 which exists on preosteoclasts' surfaces [62] and in this way has impact on osteoclastogenesis [36]. At the same time, along with TNF-α, concentration of IL-1 increases and affects degradation of cartilage matrix during its maturation into bone matrix [36].

Macrophages as *in vitro* Model in Bone Tissue Engineering

In some cases, when large bone defects occurred, bone tissue is not able to compensate the loss so it is necessary to use different bone substitutes. Bone substitutes most often include biomaterials based on tricalcium phosphates, hydroxyapatites, collagen and composites made from both inorganic and organic compounds. Also, 3D scaffolds are very useful because of their characteristic 3D structure that mimics the structure of living tissue. All of these materials can produce inflammatory reactions of macrophages *in vivo* when implanted into the defect area. Intensity of inflammation can greatly affect the course of the healing. Bearing in mind that injury itself creates local inflammatory reaction, if materials further stimulate this process, that could lead to the creation of fibrous tissue and inadequate healing process. Biomaterials that are nowadays increasingly used in bone tissue engineering are designed to have a stimulating effect on osteogenic process without having potential to induce or prolong inflammatory response of macrophages at injury site. Therefore, it is very important to show that biomaterials

are immunocompatible. For assessing the response of macrophages to different biomaterials, which can be potentially applied in bone tissue engineering and regenerative medicine, *in vitro* models of macrophages are used very often. The examination can be carried out on peritoneal macrophages, peripheral blood monocytes and different cell lines. Most commonly used cell line for these purposes is RAW 264.7 cell line. As previously stated in this paper, for the normal flow of healing process it is essential that there is a balance between M1 and M2 macrophages. It is therefore important to examine how biomaterial of interest affects the polarization of macrophages [63–65]. Another very important characteristic that biomaterials should have is to induce controlled and moderate phagocytosis by macrophages. Different *in vitro* approaches of materials testing on macrophages are used, such as direct or indirect contact assays with both direct application of materials' particles or application of materials' extracts. In both assays phagocytosis can be measured quantitatively by using standard phagocytosis tests such as NBT test [5] or Neutral red uptake test, or analyzed through materials' particles uptake assay by transmission electron microscope (TEM) [65, 66]. For assessing the production of pro-inflammatory and anti-inflammatory cytokine release from macrophages stimulated with biomaterial particles or extracts, the most frequently performed method is determination of cytokine level by ELISA assay [67–70]. For this purpose, biological assay such as L929 assay can also be used [5]. Macrophages can also be used to simulate an inflammatory state *in vitro* in order to examine how different factors released from activated macrophages can influence the osteogenic differentiation of cells [71].

Conclusion

Science progress and better understanding of pleiotropic role of macrophages in a variety of biological and pathological processes put them at the top of "cell pyramid" because of their great influence on all aspects of tissue homeostasis and tissue reparation. It is believed that these phagocytes, as well as molecules they are secreting (especially during inflammatory phase), are the key factors for the successful bone tissue repair. Future research should be directed toward modulation of macrophage's activity which might have positive influence on the final result of osteogenesis and osteoreparatory process.

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Review Article

BIOCHEMICAL AND MOLECULAR MECHANISMS OF ACTION OF CISPLATIN IN CANCER CELLS

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Abstract. *Cisplatin (cis-Diamminedichloroplatinum II) is one of the most important chemotherapeutic agents widely used in treatment of many types of solid cancer. Accumulating evidence suggests that the cytotoxic activity of cisplatin involves both nuclear and cytoplasm component, but its biochemical and molecular mechanisms of action are still unclear. Its mode of action is linked to the ability of cisplatin to interact with purine bases on the DNA, causing DNA damage, interfering with DNA repair mechanisms and inducing apoptotic cell death in cancer cells. The major limitations in the clinical application of cisplatin are the numerous side effects and the development of cisplatin resistance by tumors. Mechanisms that can explain cisplatin resistance include the reduction in drug accumulation inside the cell, higher concentration of glutathione and metallothioneins, faster repair of cisplatin adducts and modulation of apoptotic cell death in various cells. In this article we review the pathways that cisplatin can activate in cancer cell, the mechanisms of resistance and clinical toxicities. A deep knowledge of mechanisms of action may lead to design of more efficient platinum-based antitumor drugs and provide new therapeutic strategies in cancer treatment.*

Key words: *cisplatin, DNA damage, cancer cells, drug resistance, platinum-based drugs.*

Introduction

Cancer presents the second most common cause of death in Serbia, right after cerebrovascular disease. According to National cancer database cancer mortality rate is higher among men than women (181 per 100,000 men and 113.6 per 100,000 women) [1]. Lung cancer, colorectal cancer, and stomach cancer were among ten leading causes of death in men, whereas breast cancer, colorectal cancer, lung cancer, stomach cancer, and cervical cancer were among twelve leading causes of death in women [2]. Multidisciplinary approach to treatment of human malignancies includes surgery, chemotherapy or radiation therapy depending on the stage when cancer is diagnosed. Clinically useful chemotherapeutic drugs inhibit the processes essential for cancer cell growth and/or proliferation, such as blocking production of DNA, mRNA or proteins, directly damaging DNA or inhibiting components required for DNA replication or chromosome separation [3].

Cisplatin or *cis*-Diamminedichloroplatinum(II) is an effective chemotherapeutic agent that is used in nearly 50% of all cancer patients [4]. This complex was first synthesized in 1845 by Peyrone, but its antitumor activity was discovered by accident, thanks to the research of Rosenberg, the physics teacher at the University of Michigan in the late 1960s. The Food and Drug Administration approved the clinical use of this drug for treatment of genitourinary tumors in 1978, and since then it has been one of the most widely used drugs in cancer treatment [5]. It has been an important part of chemotherapeutic regimes for treatment of broad range of malignancies. Cisplatin success in treatment of testicular cancer is remarkable; its cure rate is more than 90 percent when it is used in combination with other chemotherapeutics [6]. It has been used in fight against ovarian, head and neck, bladder, cervical, esophageal, as well as small lung cancer. However, many patients eventually relapse and become refractory to the drug. Drug resistance is the major complication in cancer chemotherapy and accounts for the failure of chemotherapy to cure majority of patients. The development of platinum analogs that display similar effectiveness as cisplatin, but have better toxicity profile and lack cross-resistance is the major task in research centers worldwide.

Chemical Structure of Cisplatin

Cisplatin is a white or yellow crystalline powder, slightly soluble in water and soluble in dimethylpirimidine and N, N-dimethylformide. It is a neutral inorganic molecule with molecular weight of 301,1 g/mol, density of 3,74 g/cm^3 and melting point at 270°C, composed of platinum ion

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bound to two ammine groups and two chloride ions that are arranged in a square (Fig. 1). In metal complexes Pt can exist in either 2^+ or 4^+ oxidation state. The ammine groups represent carrier ligands, while chloride ions are leaving groups. In cisplatin the chlorides are next to each other. The presence of leaving groups is essential for biological activity of cisplatin [7]. Inside the cell, cisplatin loses two chloride ions and they are replaced by loosely bound water molecules, allowing the platinum to attack the DNA molecule in nucleus.

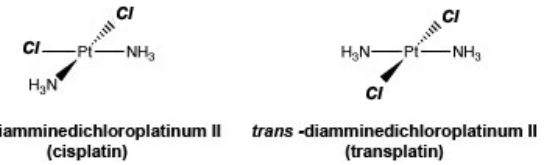

Fig. 1 Chemical structure of cisplatin and transplatin. Figure is modified from http://chemwiki.ucdavis.edu/Core/Inorganic_Chemistry/Coordination_Chemistry/Isomers/Geometric_Isomers%3A_cis-platin

Transplatin is an isomer that has both chloride ions opposite each other (Fig. 1); it causes different structural changes than cisplatin in cancer cells. Monoadducts, formed by transplatin, do not significantly change the structure and stability of DNA molecule [8].

Mechanisms of Action of Cisplatin

Cisplatin is administered to cancer patients intravenously as a sterile saline solution. In the circulation, chloride concentration is relatively high and cisplatin remains neutral and can be transported throughout the body. Once in the bloodstream, it binds strongly to plasma proteins, such as albumin and transferrin, leading to inactivation of large amount of the applied drug [9]. Passive diffusion across the plasma membrane has been proposed as process responsible for drug transport into the cell. In the last years, there is growing evidence that several proteins expressed on the cell membrane are involved in drug uptake. Copper transporter, that controls intracellular copper homeostasis, was shown to be involved in the uptake of cisplatin [10]. Many cellular components, such as cytoskeletal microfilaments, RNA and thiol-containing peptides and proteins, may react with cisplatin in the cytoplasm. Intracellular thiol-containing molecules such as glutathione and metallothionein, increase inactivation of the drug that results in cisplatin resistance.

Genomic DNA is the main cellular target for cisplatin, although only 1 percent of intracellular cisplatin is bound to nuclear DNA [11]. Cisplatin binds with DNA to form intrastrand crosslinks and adducts. DNA adducts formed by cisplatin inhibit DNA replication and/or transcription and activate several signal transduction pathways, culminating in the activation of apoptosis [12].

Cisplatin binds with DNA in two steps, first the bond with N7 guanine is formed, and then it binds with guanine or adenine in the same or opposite strand. The N7 atoms of guanine and adenine are the most accessible and cisplatin forms a broad spectrum of intra- and inter-strand crosslinks and all of them cause the distortion of the DNA. The great majority of DNA crosslinks are 1,2-d(GpG), and they represent 70 percent, while d(ApG) intrastrand adducts account for 20% of all lesions [13]. 1,2 intrastrand-crosslink is considered to be the most cytotoxic one, since inactive transplatin is not able to form this lesion. These lesions cause the bending and unwinding of the double helix and loss of function.

Several proteins can recognize the DNA bending induced by specific cisplatin adducts. High mobility group (HMG) proteins are non-histone chromosomal proteins involved in gene regulation and chromatin structure. Protein HMG1 binds with high selectivity to platinum adducts in DNA [14]. In this way, bounded proteins act as a shield and protect DNA from repair mechanisms. HMBG binding modulates signaling pathways in the cell by diminishing the efficiency of NER, and it has been connected to MMR, p53 activity and MARK pathway [15]. Recognition of 1,2-intrastrand adduct by these proteins may be the first step towards the initiation of apoptosis.

DNA lesions are recognized by damage recognition macromolecules, those can repair cisplatin DNA adducts. The most important families of DNA repair proteins are: 1) nucleotide excision repair (NER) proteins, 2) mismatch repair (MMR) proteins and 3) DNA-dependent protein-kinase (DNA-PK) proteins.

Nucleotide excision repair (NER) system consists of at least 17 different proteins. This multiprotein complex recognizes intrastrand crosslinks and subsequently excises the DNA sequences of 27-29 base pairs oligonucleotides in length containing the damage [16]. The incision reaction on both sides of the lesion involves numerous protein factors such as XPA, RPA, XRC-HR23B, ERCC1-XPF and XPG. The enzyme DNA polymerase fills the remaining gap [17]. Over-expression of some genes involved in NER complex is associated with cisplatin resistance [18]. Mismatch repair (MMR) complex is ATP dependent multiprotein system that is crucial for normal *in vivo* response to DNA damaging drugs [19]. The MMR complex causes cell cycle arrest. The MMR proteins would try to insert the correct nucleotide on the non-damaged strand opposite to the intrastrand adduct between two adjacent guanines. When it does not succeed in the attempt to repair the damage, the apoptotic pathway is activated [20]. The Ku subunit of DNA-PK protein can also interact with cisplatin-DNA lesions, which leads to the activation of DNA-PK to phosphorilate itself or other transcription factors.

Oxidative stress is one of the most important mechanisms involved in cisplatin cytotoxicity (Fig. 2). Cisplatin causes oxidative stress by increasing the level of super oxide anions and hydroxyl radicals [21]. Under oxidative stress condition, excessive reactive oxygen species (ROS) can damage cellular proteins, lipids and DNA and may modulate survival signaling cascades.

Depending on the severity and duration of ROS exposure pro-survival or pro-apoptotic response pathways may be activated. Mitochondrial glutathione (GSH) is an essential molecule in the regulation of inner mitochondrial permeability. Cisplatin decreases intracellular concentration of GSH, leading in hydroxyl radical formation and oxidative stress, resulting in loss of mitochondrial protein sulfhydryl group, calcium uptake and reduction of mitochondrial membrane potential [22]. The molecular mechanisms that underlie the cytotoxic potential of cytoplasm cisplatin may involve the pro-apoptotic Bcl-2 family members Bak1, the voltage-dependent anion channel 1 (VDAC1) and the Bak1 homolog Bax [23]. It is well known that mitochondrial DNA (mtDNA) is more susceptible than nuclear DNA to damage from reactive oxygen species, due to either a limited capacity for DNA repair or the presence of nucleosome-free structure [24]. Cisplatin is a potent mtDNA-targeting agent. Cisplatin forms crosslinks with mtDNA that is more vulnerable than nuclear DNA. The mtDNA adduct levels are higher than the nuclear DNA adduct levels, due to significantly higher number of guanine stretch sequences (target sequences of cisplatin) in mtDNA than in nuclear DNA [24].

As previously noted cisplatin inter- and intra-strand DNA adducts can be recognized and safely removed by several repair systems that normally operate in the context of a temporary cell cycle arrest. There are two main checkpoints, G1/S and G2/M, in which cell cycle will be arrested to help the function of the repair machinery. The G1/S checkpoint allows DNA restoration before replication and G2/M facilitates the reparation of DNA damaged during S and G2 phases to prevent its segregation into daughter cells. Treatment with cisplatin usually induces G2 arrest through phosphorilation checkpoint kinases Chk1 and Chk2, activation of Cdc25C and its translocation to the cytoplasm which provoke cell arrest in G2 phase of cell cycle [25]. Meanwhile, when the damage is irreparable, the cell activates mechanisms that induce cancer cell death via apoptosis and prevent the passage of these cells into mitosis. Apoptosis, as a mode of programmed cell death, is energy-dependent process leading to membrane blabbing, phosphatidylserine externalization, cell shrinkage, chromatin condensation and activation of a family of cysteine proteases called caspases [26]. There are two major pathways of apoptotic cell death: the extrinsic pathway, activated by pro-apoptotic receptor signals at the cell surface, and the intrinsic pathway, activated by mitochondrial signals. In response to DNA damage, the Bcl2 family proteins regulate apoptosis through cytochrome c, apoptosis promoting activating factor 1 (Apaf-1) and caspases 9 and 3.

It is known that p53 protein plays a central role in chemotherapy-induced apoptosis. A primary mechanism by which p53 induces apoptosis is through transcriptional activation and repression of target genes whose promoters contain p53-binding sites. These genes may activate apoptotic process via multiple pathways (Fig. 2) [27]. The protein p53 is "guardian of the genome" because it activates a host of other genes (p21/waf1, mdm2, GADD45 and others) that lead to cell cycle arrest and activation of DNA repair [28]. On the other hand, p53 regulates cisplatin-induced apoptosis by several mechanisms like: activation of pro-apoptotic genes including PUMA [29], caspases [30], PIDD [31], MAPK protein family [32], as well as interaction with Bcl2 family proteins in mitochondria and/or cytosol

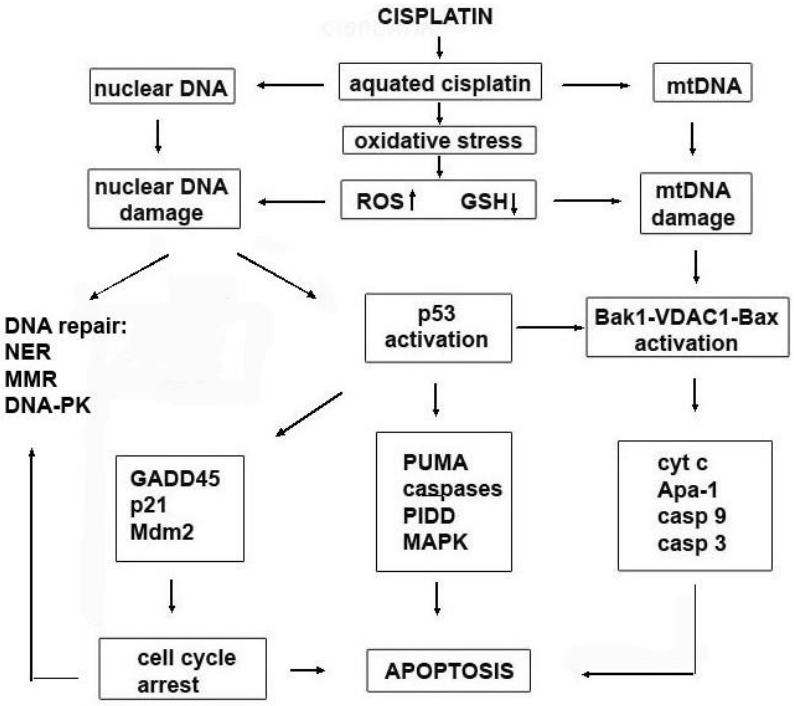

Fig. 2 Molecular mechanisms of cisplatin in cancer treatments

[33]. The p53-negative cells also respond to cisplatin-induced DNA damage that suggests the existence of alternate pathways upon the stress.

Side Effects of Cisplatin

Chemotherapy is associated with increased toxicity, especially in older patients. The efficiency of cisplatin administration is often limited by its side effects. Various studies confirmed that cisplatin induces the formation of ROS, responsible for the numerous side effects like nephrotoxicity, ototoxicity, hepatotoxicity, cardiotoxicity and neurotoxicity.

The kidney is the main route for excretion of cisplatin and it accumulates it to a greater degree than other organs, which is the reason for the cisplatin-induced nephrotoxicity. Tubular cell injury occurs in one third of cisplatin treated patients and manifests as an increase in serum urea and creatinine concentration and imbalanced electrolytes [34]. Proximal tubulocytes are the main point of cisplatin action. The concentration of the drug in these cells is five times higher than its serum concentration [35]. Pathological changes are most prominent in S3 segment and they are caused by multiple mechanisms such as oxidative stress, apoptosis, inflammation and fibrogenesis. Nephrotoxicity is cisplatin-dose-dependent [36]. Adequate hydration can decrease the reactive monohydrated cisplatin form and it is renoprotective.

Cisplatin is the most ototoxic drug known. Between 10 and 90 percent of treated patients develop some degree of hearing loss. These changes are irreversible and pediatric population is very vulnerable [37]. The destruction affects auditory sensory cells in the organ of Corti and both hearing and vestibular functions can be affected [38]. Ototoxicity is irreversible and it is associated with hipoalbuminemia, application of other medicaments, genetic factors, renal failure, and patient's age [39]. Otoprotective therapy should be administrated. Intratympanic application of the drug is the most effective, without compromising antitumor effect.

High dose of cisplatin may cause hepatotoxicity. Oxidative stress appears to play an important role in cisplatin-induced hepatotoxicity liver injury [40]. Cisplatin therapy has been associated with mild elevation of transaminases and bilirubin in circulation [41]. Recent studies show that administration of high doses of selenium and vitamin E has protective effect on liver injury [42]

Antineoplastic therapy with cisplatin induces lipid peroxidation of cardiac membranes leading to serum elevation of lactate dehydrogenase and creatine kinase. Arrhythmias and prolongation of QT-interval have been reported in vulnerable individuals [43].

Cisplatin is thought to act on the dorsal root ganglion to generate both transient and chronic neuropathies, which explain the primary sensory neuropathy commonly observed in patients treated with cisplatin [44]. Anti-oxidant compounds are being developed to prevent these toxic side effects.

Cisplatin administration results in side effects common to most cytotoxic agents such as nausea, vomiting, myellosuppression, gastrotoxicity and some reproductive toxic effects [45].

Development of Cisplatin-induced Resistance

Tumor cell resistance to chemotherapeutic drugs is a barrier to improving outcomes in these patients. Cisplatin resistance is a multifactorial phenomenon and may include changes in cellular uptake, decreased influx or increased efflux of drug, glutathione or metallothionein conjugation or drug detoxification. The increased DNA repair and inhibition of apoptosis is the significant mechanism of resistance. The resistance can be intrinsic, in which the drug is ineffective from the onset or acquired resistance, in which a drug is initially beneficial but becomes ineffective over time [46].

Reduced drug accumulation is predominantly caused by defect in the uptake of a drug. It has been further confirmed in human ovarian carcinoma cell line that cisplatin, at plasma concentration, rapidly downregulates protein expression of Ctr1 [47]. Two other copper transporters have also been implicated in resistance to cisplatin: ATP7A and ATP7B. These copper transporters are responsible for the export of copper from the cell. High levels of ATP7A and ATP7B expression lead to cisplatin resistance [48].

In the cytoplasm aquated cisplatin reacts with thiol containing compounds including glutathione and metallothioneins. Glutathione-S-transferase catalyses the reaction where cisplatin is conjugated with glutathione and therefore, cisplatin can not bind with DNA and other cellular targets. In some malignant tissues, there is a positive correlation between resistance to treatment and cellular level of glutathione as well as over expression of GST and other enzymes involved in glutathione metabolism [49, 50]. Metallothioneins, a family of low molecular weight thiol-rich proteins, can bind cisplatin in cytoplasm leading to drug inactivation in some tumor cell lines [51, 52, 53].

Alterations of the DNA repair pathways are important for mediating resistance. Studies of testicular and ovarian carcinoma cell lines showed a deficiency in NER mechanism in cells that were sensitive to platinum therapy [54, 55]. The NER is the main repair pathway that involves recognition of the damage and incision that requires various proteins including ERCC-XPF. The level of ERCC1 protein inversely correlates with the response to chemotherapy in gastrointestinal and non-small cell lung carcinoma [56, 57].

Resistance mechanisms, therefore, arise as a consequence of intracellular changes that either prevent cisplatin from interacting with DNA, interfere with DNA damage signals for activating the apoptotic machinery, or both. More than one mechanism is usually observed in resistant cells, and this contributes to the multifactorial nature of cisplatin resistance. To minimize cisplatin resistance, combinatorial therapies were developed and

have been proven to be more effective in defeating cancer. The main goal is to find compounds that are less toxic, have no cross-resistance and possibly are more efficient than cisplatin. Drug resistance is the single most common reason for discontinuation of the drug.

Development of New Platinum-based Antitumor Drugs

Different modifications of cisplatin have been investigated in order to obtain a drug that has better toxicity profile and wider therapeutic spectrum than cisplatin. In order to reduce toxic side effects and overcome cancer cell resistance, new platinum drugs have been developed. Although a large number of platinum compounds underwent *in vitro* testing, less than a thirty entered clinical trials [58].

Cisplatin, carboplatin and oxaliplatin (Fig. 3) are worldwide approved drugs that have a major role in human oncology.

The second generation platinum drug carboplatin was introduced into cancer therapy in 1989, for treatments of ovarian cancer. The replacement of the chloride groups of cisplatin by cyclobutanedicarboxylate ligand of carboplatin (Fig. 3) provides good aqueous solubility and greater stability and leads to diminishing side effects. Carboplatin can be applied in higher doses with possibly better effects. The downside is that carboplatin and cisplatin are cross resistant.

Newly acquired knowledge about mechanism of tumor resistance to platinum drugs enabled discovery of third generation drugs such as oxaliplatin that is effective in colon cancers, which were thought to be resistant to platinum compounds. Oxaliplatin has a different carrier ligand diaminocyclohexane (DACH) [59], that has less cross-resistance and a more favorable toxicity profile.

Satraplatin, lipophilic platinum (IV) complex is the first platinum compound active after oral administration and is currently in different phases of clinical research [60]. Platinum (IV) complexes are less reactive in ligand substitution reactions compared to their platinum (II) analogues, and they have reduced toxicity and a smaller fraction of the drug deactivated on its pathway to target cell [61]. The platinum (IV) complexes are in focus and they have been tested in various cancer cell lines [62, 63]. Newly synthesized platinum (IV) complexes are tested for cytotoxic activity against various cell lines and some of them showed similar activity as cisplatin towards human ovarian carcinoma, breast cancer and colon carcinoma cell lines [64, 65].

Picoplatin is platinum coordination complex which, during in vitro testing, showed activity against several cisplatin-resistant and oxaliplatin-resistant cell lines. Unfortunately, it failed to produce significant clinical results compared to standard therapy for lung cancer [66].

Multinuclear complexes are another class of platinum complexes that showed activity in both cisplatin resistant and cisplatin sensitive cell lines. They are di-, three-, or tetra-nuclear compounds, in which platinum centers are connected by rigid or flexible bridges [67, 68]. The DNA binding of these compounds is structurally different from binding of cisplatin and its analogues and they exhibited cytotoxicity in cancer cell models, and some of them entered clinical trails [69].

Platinum drugs resistance can also be circumvented by improved delivery of the drug to tumor tissue. This can be achieved by linking platinum-based drug to a water soluble, biocompatible co-polymer [70]. In some cases, such as an ovarian cancer, local application of a drug, through intraperitoneal injection might be adequate [71].

Conclusions

Cisplatin plays a major role in the treatment of a variety of malignances. Cisplatin and other platinum-based compounds are cytotoxic drugs which kill cancer cells by damaging nuclear and mitochondrial DNA, inhibiting DNA replication and mitosis and inducing apoptotic cell death. Cisplatin-induced damages are considered to be an important trigger of p53 activation that leads to cell apoptosis. On the other hand, cisplatin can also react with other cellular components such as membrane phospholipids and proteins, cytoskeletal microfilaments, thiol-containing biomolecules and cytoplasm proteins, resulting in cell death depending upon the mechanism of DNA damage. Unfortunately, the therapeutic effects of cisplatin are often limited due to cell resistance which develops through changes in drug transport, detoxification, DNA repair and apoptosis signaling pathways. Dose dependent toxicity and acquired and intrinsic resistance are still the major obstacles in platinum based therapy. Therefore, the comprehensive understanding of the mechanisms of action and tumor resistance might be useful in defining new strategies in the search for the new therapeutics with improved pharmacological properties.

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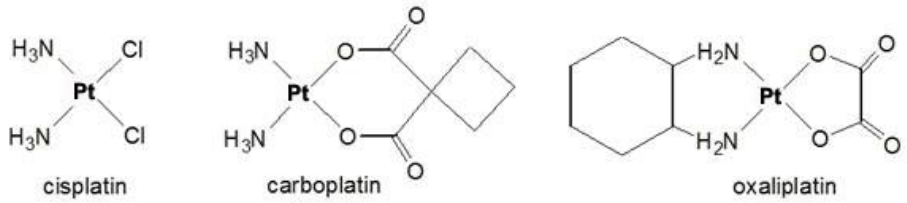

Fig. 3 Chemical structures of cisplatin, carboplatin and oxaliplatin. Figure is modified from http://www.dscf.units.it/ricerca_grp.php?name=inorgani1group&menu=research&file=ruthenio/ruthenio

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Original Article

COMPARISON OF TREATMENT OUTCOME AMONG PATIENTS WITH CHRONIC MYELOID LEUKAEMIA WHO ACHIEVED COMPLETE CYTOGENETIC RESPONSE WITHIN OR AFTER ONE YEAR OF IMATINIB MESYLATE THERAPY

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Abstract. *The introduction of imatinib, as a type of targeted molecular therapy, has profoundly changed the treatment outcome of chronic myeloid leukaemia (CML). The aim of this study was to assess and compare treatment outcome among patients who achieved complete cytogenetic response (CCgR) within or after one year following initiation of imatinib therapy. A group of 42 adult patients with early chronic-phase Philadelphia-positive CML treated with imatinib mesylate therapy has been studied. In the study group CCgR has been achieved in 36/42 (85.71%) analysed patients, while in 3/42 (7.14%) patients the absence of cytogenetic response has been noted. Early CCgR has been achieved by 25/36 (69.44%) patients with response at median time of 6.9±1.9 months, while late CCgR has been achieved by 11/36 (30.56%) patients at median time of 18.75±2.4 months. Univariate analysis has identified prognostic factors for achieving early and late CCgR. Analysis of remission duration of treatment responders has shown that 21/25 (84%) patients in the group with early CCgR and 9/11 (81.81%) patients from the group with late CCgR still maintained stable remission on last cytogenetic control. The estimated 5-year survival rate was 85% for early responders and 74% for late responders. In conclusion, these results demonstrate that there are no differences in the treatment outcome, i.e. level of response, of patients with CML in relation to whether the CCgR was achieved within or after one year of imatinib therapy*.

Key words: *chronic myeloid leukaemia, imatinib, prognostic factors, treatment outcome.*

Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disease that occurs because of constitutive activation of the BCR-ABL tyrosine kinase, a result of the t(9;22) (q34;q11) translocation designated as the Philadelphia (Ph) chromosome [1]. The introduction of the tyrosine kinase inhibitors (TKIs), as a type of targeted molecular therapy, has revolutionized the management and outlook in CML [2]. The largest study up to date that provides the data on the effectiveness of imatinib in CML patients is IRIS study. It has shown that when imatinib was given as an initial treatment of patients in early chronic phase CML, complete hematologic response (CHR) after one year occurred in 95% of patients and complete cytogenetic response (CCgR) in 76%. Of CML patients who achieved a CCgR, major molecular response (MMoR) was achieved by 57%. After 5 years of treatment, the estimated rate of progression-free survival was 84%, and an estimated 93% of patients had not progressed to the accelerated phase or blast crisis [3]. Initial studies have shown the importance of early achievement of therapeutic response, not only achievement of CCgR but also MMoR and particularly within the first year of therapy, what has been predictive of durable cytogenetic remission [4, 5]. Similarly, according to achieving CCgR or not at 12 months, the 3-year event free survival rate was 98% and 67%, and overall survival was 99% and 94% [6]. However, another study [7] has shown that there was no difference between group of patients with early and late achievement of CCgR according to progression-free survival rate and an estimate 4-year overall survival (100% vs. 88% and 100% vs. 92%, respectively). Thus it has been demonstrated that it was important to achieve CCgR, and that the time of achieving this level of response was of less importance.

The aim of this study was to compare two groups of patients with early and late CCgR to determine whether there are differences in treatment outcome compared to when CCgR was achieved. To explore the difference between the two groups based on the time when patients achieved CCgR, one year was chosen as the cut-off point. This paper presents the examination of the

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connection between the characteristics of patients before treatment and the subsequent possibility of achieving early and late CCgR, in order to determine which baseline characteristics of patients lead to differences in treatment outcome. The probability of maintaining CCgR and survival in all patients and in groups with early and late CCgR has also been determined.

Material and Methods

The analysis included 42 adult patients who were treated in our institution with the diagnosis of Ph-positive CML in early chronic phase of the disease, during the period from 2006 to 2014. In this study patients with CML who achieved CCgR to imatinib therapy have been analysed. Patients were divided into two groups: the patients who achieved CCgR within 1 year (early response) and patients in whom CCgR has been achieved after 1 year from the beginning of treatment (late response). The study analyses the treatment response and survival rate of patients in these two groups in order to determine whether there are differences in relation to when CCgR was achieved.

The patients have not received prior therapy for leukaemia except hydroxyurea which has been conducted for initial leukoreduction. All patients started treatment with recommended oral dose of imatinib of 400mg once a day. Escalated doses of 600mg and 800mg were administered in case of failure of previous treatment, apropos in patients with cytogenetic relapse or cytogenetic refractoriness.

Chronic phase CML was defined according to the recommendations of the LeukaemiaNet panel [8, 9] as the presence in the peripheral blood of blasts less than 15%, basophils less than 20%, blasts together with promyelocytes less than 30%, and platelets more than 100×10^9/L. After the start of treatment haematological and cytogenetic responses have been evaluated in order to monitor the response to the treatment. Complete blood count and serum chemistry evaluations have been performed every month until the CHR was achieved, and then every 6 months or in accordance with other controls. Marrow studies, including morphologic and cytogenetic analysis have been performed every 6 months to 2 years of therapy, and then every year in terms of disclosure of additional chromosomal aberrations in case they have achieved stable CCgR. Cytogenetic response has been assessed by conventional cytogenetics with direct preparation of material from the bone marrow with optimal number of mitosis of at least 20 for assessing response. The response criteria have also been defined according to recommendations of the LeukaemiaNet panel [8, 9]: complete hematologic response (CHR) has been defined as a white blood cell count of less than 10×10^9/L, a platelet count of less than 450×10^9/L, the absence of immature cells (blasts, promyelocytes, myelocytes) in the peripheral blood, and disappearance of all signs and symptoms associated with leukaemia (including palpable splenomegaly) for at least four weeks. Cytogenetic response has been defined as: complete 0% Ph+ cells in metaphase, partial 1%-35% Ph+ cells in metaphase, minor 36%-65% Ph+ cells in metaphase, minimal 66%-95% Ph+ cells in metaphase and absent >95% Ph+ cells in metaphase. Major cytogenetic response (MCgR) included complete plus partial cytogenetic response.

The results are presented in tables and graphs, processed according to the methodology of descriptive and analytical statistics. Standard descriptive statistical methods (number, proportion, mean, range) have been used to summarize the characteristics of the patients before treatment and for monitoring the cytogenetic response to therapy. To identify potential prognostic factors associated with early and late CCgR Pearson χ^2 test has been used. The following levels of statistical significance of Pearson χ^2 test have been used: n.s. without statistical significance, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. For the evaluation of the probability of survival Kaplan-Meier method has been used.

Results

A total of 42 adult patients with newly diagnosed Ph-positive early chronic phase CML who were treated with imatinib have been analysed. The average age of patients was 50.52 years (range, 19-73 years) and 22 patients (52.38%) were female. Significant characteristics of the patients before treatment with imatinib are presented in Table 1. Median duration of disease from diagnosis to initiation of treatment with imatinib therapy was 2.5 months (range 1-7 months). The mean follow-up of patients in this study was 48.4 months (range 32-90 months) and none of the patients were lost during follow-up.

The rate of cytogenetic response, stability and duration of CCgR during the entire study period are shown in Table 2. In the study group CCgR has been achieved in 85.71% of the analysed patients, while in 7.14% of patients the absence of cytogenetic response to imatinib after more than 1 year of treatment has been noted. Patients who achieved CCgR were divided into two groups according to the time needed to achieve this level of response. Group with early CCgR comprised 69.44% of the total number of analysed patients, with a median time of 6.9±1.9 months (range 3-12 months) needed to accomplish this response. In this group 88% of patients achieved CCgR at 6 months, while 12% of patients achieved the same response between 6 and 12 months. Group with late CCgR comprised 30.56% of the patients, and the median time from start of treatment until CCgR was accomplished was 18.75±2.4 months (range 15-24 months). In this group, 81.82% achieved CCgR at 18 months and 18.18% between 18 and 24 months of treatment with imatinib. Analysis of steadiness of CCgR has shown that 84% of the patients in the group with early CCgR and 81.81% of the patients in the group with late CCgR still maintained stable remission without elements of clonal cytogenetic progression during

the last control. These results show that there was no difference between the groups of patients with early and late response in terms of loss of already achieved CCgR.

Table 1 Clinical and laboratory characteristics of patients with chronic myeloid leukaemia before treatment with imatinib

Parameter	Mean values±SD, (range)	
Age, g	50.5±13.8	(19–73)
Time from diagnosis to imatinib, m	2.5±1.7	(1–7)
WBC count $\times 10^9$/L	114.6±73.8	(20–298)
Platelets $\times 10^9$/L	395.5±230.8	(140–1165)
Haemoglobin, g/dL	118.5±19.5	(74–145)
Peripheral blasts, %	2.0	(0–7)
Peripheral basophils, %	2.8	(0–9)
Marrow blasts, %	3.0	(0–6.5)
Marrow basophils, %	3.4	(0–10)
Splenomegaly, n (%)	29	(69.1)
Dose, mg, n (%)		
400	30	(71.4)
600	5	(11.9)
800	7	(16.7)
Sokal score, n (%)		
Low	20	(47.7)
Intermediate	19	(45.2)
High	3	(7.1)
Hasford score, n (%)		
Low	27	(64.3)
Intermediate	12	(28.6)
High	3	(7.1)

Table 2 The rate of cytogenetic response to imatinib therapy in the analysed period

	Patients	
Cytogenetic response (CgR)	Number (n)	%
Complete CgR,	36/42	85.71
Early Complete CgR,	25/36	69.44
Late Complete CgR,	11/36	30.56
Early response and maintenance Complete CgR	21/25	84.00
Late response and maintenance Complete CgR	9/11	81.81
Partial to minimal CgR	3/42	7.14
Absent CgR	3/42	7.14

Correlation between the basic characteristics of the patients and the subsequent possibility of achieving early CCgR have been analysed and presented in Table 3. According to statistical analysis out of 12 baseline variables 5 of them were identified as prognostic factors for achieving early CCgR: less than 5% of marrow blasts, less than 5% of marrow basophils, less than 4% of peripheral basophils, the absence of peripheral blasts ($p < 0.001$), as well as low Hasford risk score ($p < 0.05$).

Table 3 Prognostic factors associated with early complete cytogenetic response

Characteristics	n	Early CCgR	p	
Age (years)				
<60	31	15 (48.39)	0.114	n.s.
≥60	11	10 (90.91)		
Haemoglobin (g/dL)				
<10	16	10 (62.50)		
10–11.9	13	9 (69.23)	0.465	n.s.
≥ 12	13	6 (46.15)		
WBC count (x 10^9/L)				
<50	9	6 (66.67)		
50–99	15	7 (46.67)	0.449	n.s.
≥ 100	18	12 (66.67)		
Platelets (x 10^9/L)				
<450	21	14 (66.67)		
450–699	16	8 (50.00)	0.592	n.s.
≥ 700	5	3 (60.00)		
Peripheral blasts (%)				
0%	10	10 (100.0)		
1–2%	22	13 (59.09)	0.001	***
≥ 3 %	10	2 (20.00)		
Marrow blasts (%)				
< 5%	36	25 (69.44)	0.000	***
≥ 5%	6	0 (0.00)		
Peripheral basophils (%)				
< 4%	27	21 (77.78)	0.001	***
≥ 4%	15	4 (26.67)		
Marrow basophils (%)				
< 5%	33	25 (75.76)	0.000	***
≥ 5%	9	0 (0.00)		
Splenomegaly (bcm)				
0	13	10 (76.92)		
1–9	21	12 (57.14)	0.193	n.s.
≥ 10	8	3 (37.50)		
EUTOS score				
Low	40	25 (62.50)	0.079	n.s.
High	2	0 (0.000)		
Hasford score				
Low	27	19 (70.37)		
Intermediate	12	6 (50.00)	0.045	*
High	3	0 (0.00)		
Sokal score				
Low	20	12 (60.00)		
Intermediate	19	13 (68.42)	0.081	n.s.
High	3	0 (0.00)		

*p<0.05 **p<0.01 ***p<0.001
n.s. without statistical significance, bcm-below costal margin

Baseline characteristics of patients that are associated with the achievement of the late CCgR are shown in Table 4. Of the 12 pre-treatment characteristics only two have been identified as prognostic factors for achieving late CCgR: the presence of more than 3% of peripheral blasts and value of haemoglobin less than 10g/dL ($p < 0.05$).

Table 4 Prognostic factors associated with late complete cytogenetic response

Characteristics	n	Late CCgR	p	
Age (years)				
<60	31	11 (35.48)	0.121	n.s.
≥60	11	0 (0.00)		
Haemoglobin (g/dL)				
<10	16	1 (6.25)		
10-11.9	13	3 (23.08)	0.014	*
≥ 12	13	7 (53.85)		
WBC count (x 10^9/L)				
<50	9	2 (22.22)		
50– 99	15	7 (46.67)	0.066	n.s.
≥ 100	18	2 (11.11)		
Platelets (x 10^9/L)				
<450	21	4 (19.05)		
450–699	16	6 (37.50)	0.425	n.s.
≥ 700	5	1 (20.00)		
Peripheral blasts (%)				
0%	10	0 (0.00)		
1–2%	22	6 (27.27)	0.039	*
≥ 3 %	10	5 (50.00)		
Marrow blasts (%)				
< 5%	36	9 (25.00)	0.667	n.s.
≥ 5%	6	2 (33.33)		
Peripheral basophils (%)				
< 4%	27	5 (18.52)	0.129	n.s.
≥ 4%	15	6 (40.00)		
Marrow basophils (%)				
< 5%	33	8 (24.24)	0.582	n.s.
≥ 5%	9	3 (33.33)		
Splenomegaly (bcm)				
0	13	3 (23.08)		
1–9	21	6 (28.57)	0.936	n.s.
≥ 10	8	2 (25.00)		
EUTOS score				
Low	40	10 (25.00)	0.433	n.s.
High	2	1 (50.00)		
Hasford score				
Low	27	6 (22.22)		
Intermediate	12	4 (33.33)	0.735	n.s.
High	3	1 (33.33)		
Sokal score				
Low	20	6 (30.00)		
Intermediate	19	4 (21.05)	0.783	n.s.
High	3	1 (33.33)		

*p<0.05 **p<0.01 ***p<0.001 n.s. without statistical significance, bcm-below costal margin

Of the 42 analysed patients 36 are alive, six patients died, two in accelerated phase and four due to complications with associated diseases. In case of 30 patients stabile CCgR on the last cytogenetic control was maintained and they have been on therapy with imatinib. Twelve patients were excluded from imatinib therapy, nine were treated with second-generation of tyrosine kinase inhibitors, three were treated with interferon plus cytarabine.

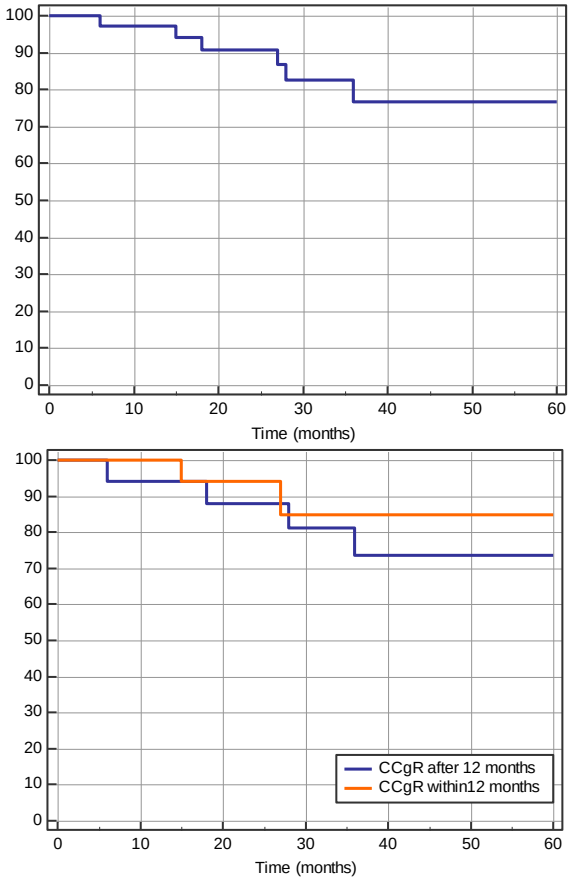

Fig. 1 Kaplan-Meier survival probability in all analysed patients (A) and in patients who have achieved complete cytogenetic response within 12 months or later during treatment with imatinib (B).

Finally, in this study the survival probability has been determined for all patients, both in groups with early and late CCgR. Figure 1 shows the estimated 5-year survival rate for all patients with CCgR (A) and (B), namely for patients who achieved this response within 1 year or later during treatment (n=25 for early response and n=11 for the late response). After 5 years of treatment, 77% of patients with CCgR were still alive (Fig. 1A). The estimated 5-year overall survival rate was 85% in patients with early response and 74% for patients with late response (Fig. 1B).

Discussion

The introduction of imatinib in CML therapy, a potent and selective inhibitor of tyrosine kinase, has led to great progress in the treatment of these diseases [10]. Most of the available data on the efficacy of imatinib in patients with CML is based on the results of the IRIS study. This study showed that after an average follow-up of 54 months, the rate of CHR was 93%, CCgR was 89%, while the rate of absence of progression was 91%. IRIS study has reported the significance of achieving cytogenetic response at 12 and 18 months, and that the early response at 3 and 6 months was also important

[11]. A six-year update of the IRIS study showed that the best cumulative rate of CCgR was 82% and the estimated overall survival rate after 6 years was 88%-95% [12]. Results of 8-year IRIS study update [13] once again confirmed long-term efficacy and safety of imatinib.

Clearly, it had been established that the depth of response to imatinib therapy was the most important prognostic factor for treatment outcome of patients with CML [14]. Initial reports suggested the importance of achieving cytogenetic response and showed that patients without cytogenetic response at 6 months and those with minimal cytogenetic response at 12 to 18 months, had a worse estimated 4-year survival rate of 70% and 79%, compared to those who had a better cytogenetic response in whom the estimated 4-year survival rate was 88% and 100% [15]. Similarly, subsequent analysis has again confirmed that the cytogenetic response to imatinib at 12 months is indicative of a prognosis. Patients who did not achieve CCgR plus PCgR at 12 months had worse estimated 3-year survival rate than the rest, 84% versus 99%. The estimated 5-year survival rate for patients achieving CCgR and PCgR at 12 months was 94% in the both cytogenetic subgroups [16]. Analysis of steadiness of CCgR in the study of Iacobucci I. et al. [7] showed that in the group of patients who achieved CCgR at 12 months, 81% of patients continued to maintain stable CCgR at 48 months of follow-up, while 19% of patients showed a loss of response in the same period. It has also been demonstrated that there was no difference between the groups of patients with early and late response in terms of CCgR loss. Similar results were also obtained in this study: in patients with early response 84% were still in stable CCgR at the last cytogenetic control, while 16% of patients showed loss of cytogenetic remission. Our data indicate that patients with late response maintained stable cytogenetic response of treatment in 81.81%, while 18.19% of patients lost CCgR during follow-up.

Considering that failure regarding achieving CCgR at 12 months of therapy was associated with a higher risk of disease progression, Cardema et al. [17] have analysed factors associated with achieving early response. Univariate analysis identified the following characteristics of patients prior to treatment to be independent poor prognostic factors for achieving early CCgR: lower haemoglobin, higher percentage of blasts in the peripheral blood and bone marrow, splenomegaly and imatinib therapy in the standard dose. In the multivariate analysis, lower haemoglobin, higher percentage of blasts in the peripheral blood and treatment with standard dose imatinib, remained as predictors of a decreased opportunities for achieving early CCgR on imatinib therapy. In this study the results of the correlation between the basic characteristics of the patients and the subsequent possibility of achieving early CCgR were to some extent different. This analysis showed that 5 variables were significant predictors of achieving early CCgR: lower marrow blasts, lower marrow basophils, lower peripheral basophils, the absence of peripheral blasts ($p<0.001$), as well as low Hasford risk score ($p<0.05$).

In this study, analysis of the estimated 5-year survival has shown that these probabilities in patients with early and late CCgR were not significantly different. These findings correlate with the results of several studies which have confirmed a cytogenetic response being important prognostic factor for long-term outcome of patients with CML. Five-year update of the IRIS study showed that progression-free survival was better for patients who achieve CCgR regardless of whether that response was achieved at 12, 18 or 24 months, which indicated that the time of achieving cytogenetic response was of the lesser importance [3]. Similarly, it has been shown that patients treated with imatinib who achieved CCgR at 12 months of treatment had progression-free survival rate and the estimated 4-year survival rate similar to those who have not achieved CCgR at 12 months [7]. It is worth mentioning that there are also reports that showed that among patients who achieved CCgR significant differences were not observed in the duration of CCgR and disease-free survival regardless of whether the CCgR has been achieved within or after 12 months of imatinib therapy. Although patients who did not achieve CCgR can improve response during continued imatinib therapy, they basically have two options: either to achieve CCgR or progress to acceleration phase [17].

Conclusion

Results presented in this paper suggest that among patients who achieved CCgR there is no significant difference in the rate and duration of CCgR regardless of whether they belong to a group with early or late response. Analysis of the estimated 5-year survival for patients with CCgR has shown similar results in early and late responders. All these results indicate that there is no difference in the treatment outcome of patients with CML in relation to whether the CCgR was achieved within or after one year of imatinib therapy.

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Original Article

MODERN MANAGEMENT OF THYROGLOSSAL DUCT CYST

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Abstract. *Thyroglossal duct cysts (TGCs) represent the most common congenital anomaly of the neck (7% of the population). They account for 2–4 % of all neck masses, and 70% of congenital neck abnormalities. Over half of cases are present in the first decade of life but they may also be seen in adults. Pyramidal lobe of the thyroid is the most common remnant of the thyroglossal tract and if no other thyroid tissue is identified, patients require lifelong replacement therapy after removal. TGCs arise from a persistent epithelial tract formed with the descent of the thyroid from the foramen caecum to its final position in the front of the neck. This duct obliterates early in fetal life. The duct so formed can rise in sinuses, fistulae or cysts. Symptoms can arise from the swelling itself or from complications, the most significant of which is infection. Surgical treatment of choice for TGCs is Sistrunk operation which includes dissection of the hyoid bone to the base of the tongue. Cancer has been reported in a small number of patients in whom the cyst is not removed until adulthood. Further studies are required to promote and establish novel treatment techniques, especially for recurrent cases.*

Key words: *thyrogossal duct cyst, diagnosis, treatment*.

Introduction

Thyroglossal duct cysts (TGCs) represent the most common congenital anomaly of the neck (7% of the population). They account for 2–4 % of all neck masses, and 70% of congenital neck abnormalities.

TGCs arise from a persistent epithelial tract formed with the descent of the thyroid from the foramen caecum to its final position in the front of the neck. This duct obliterates early in fetal life.

The duct so formed can rise in sinuses, fistulae or cysts.

Symptoms can arise from the swelling itself or from complications, the most significant of which is infection [1–8].

Objective

To review and discuss the management options of thyroglossal duct cysts.

Methods

Analysis of databases was performed to identify the relevant articles following a thematic qualitative analysis. Due to the variability of treatments along with the scarce amount of evidence, neither a formal systematic review nor a meta analysis were considered to be manageable. A qualitative analysis using a common thematic coding was performed instead, and clinical narrative review is presented.

Histology

Thyroglossal duct cyst is a well defined cyst with an epithelial lining composed of ether squamous or respiratory epithelium.

There can sometimes be an island of thyroid tissue lying in the walls of the cysts. Thompson et al. reported that thyroid gland tissue is identified in 71 % of cases (0.45 cm mean size), although not limited to the cyst wall, but present in the surrounding soft tissues. Cysts are filled with mucoid of muco-purulent material depending on whether the cyst has been infected.

Types of Thyroglossal Duct Cysts

- Infrahyoid type – 65% and is mostly found in the paramedian position.
- Suprahyoid type – nearly 20% and is positioned in the midline.
- Juxtahyoid cyst –15%.
- Intralingual location – 2%.
- Suprasternal variety – 10% of cases.
- Intralaringeal – very rare.

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Embriology

The thyroid gland, although situated in the lower position of the neck around the trachea, originates in the mouth at the back of the tongue and then moves down the neck during development [1–5]. As the thyroid gland moves down to its normal position, there is connection to the base of the tongue (Fig. 1). That should disappear by the time the thyroid reaches its final position. If it does not, there may be a persistent hallow tube that may allow accumulation of mucoid material and the formation of a cyst at the end.

This is known as a thyroglossal duct cyst. Frequently this is noted soon after a cold when there has been swelling of the tonsils and others lymphoid tissue of the throat [7–9].

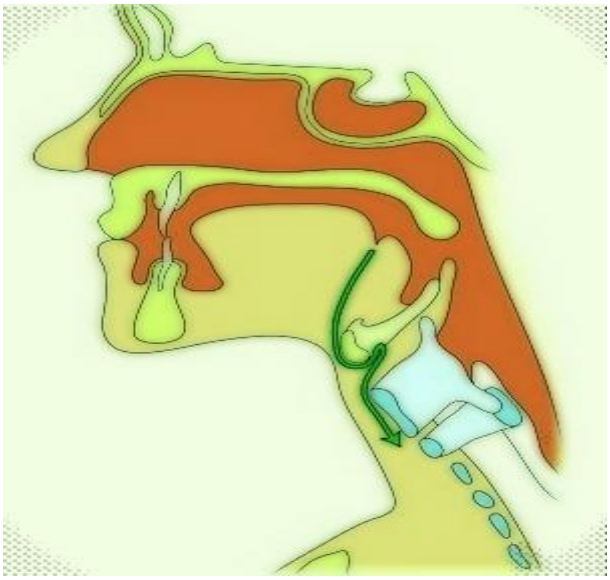

Fig. 1 Thyreoglossal duct

Diagnosis

Approximately three-quarters of thyroglossal duct abnormalities present as cysts, whereas 25% present as a draining sinus on the skin. A sinus occurs as a result of infection (in the cyst) and rupture onto the chin with persistent drainage. The cysts are generally asymptomatic and are noticed by the family as a soft swelling under the skin over the area of the hyoid bone, a floating bone in the upper neck to which the tongue muscles are partially attached.

TGCs are usually single, smooth and 1–3 cm in size and move when the patient swallows or protrudes the tongue. The other causes for masses in this area of the neck include abnormally located thyroid tissue, lymph nodes, and dermoid cyst.

Imaging

X-rays are not usually needed as the diagnosis is frequently made by examining the mass. Thyroid scanning is not generally necessary but is reserved for patients who have either no detectable thyroid tissue in the neck on examination, or who following surgery have thyroid tissue noted within the surgical specimen. The tissue in this abnormal location is sometimes removed, and if no other thyroid tissue is identified, patients require lifelong replacement therapy [10–12].

They can be diagnosed with multiple imaging modalities including ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI).

Ultrasound and CT are radiologic tools of choice. US is the gold standard and it can distinguish between solid and cystic components (Fig. 2).

CT and contrast CT (MSCT) may reveal a well circumscribed cystic lesion, 2–4cm in diameter with capsular enhancement [12].

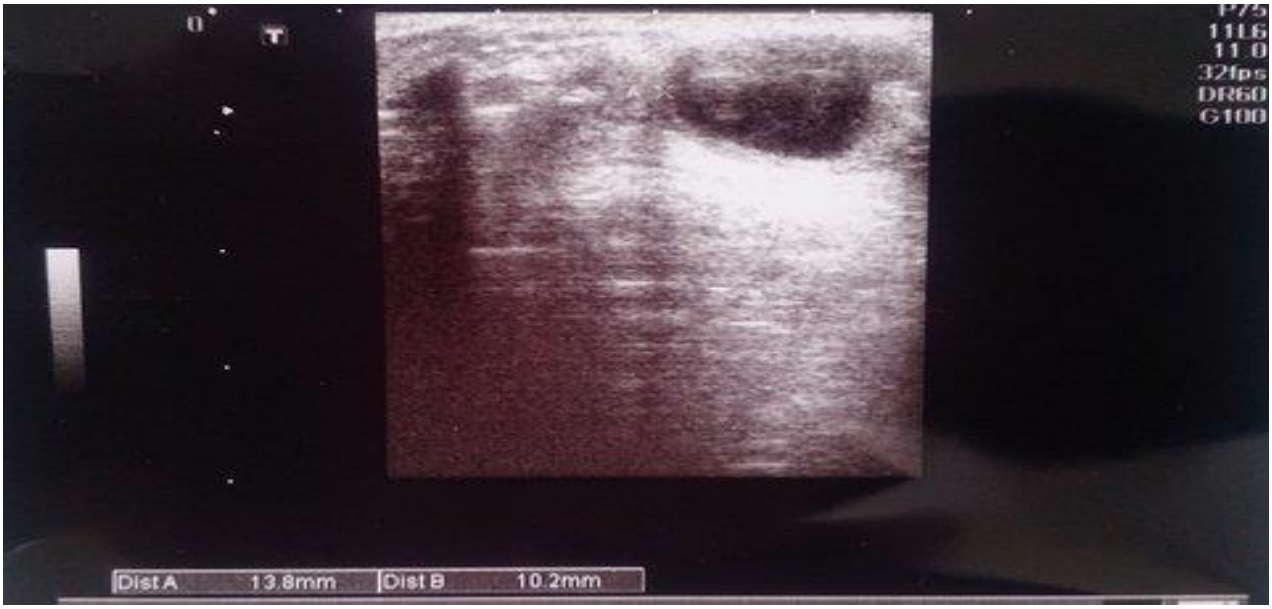

Fig. 2 Ultrasound of TGC, 21 years old female

Clinical Appearance

- Non tender and mobile masses.
- Infected cyst may manifest as tender mass with dysphagia, dysphonia, draining sinus fever and enlarging neck mass.
- Often appear with respiratory tract infection.
- Airway obstruction is possible, especially with intralingual cysts.
- The pathognomonic sign is that the cyst moves with tongue protrusion.

Differential Diagnosis

- Dermoid cyst
- Lymphadenopathy
- Sebaceous cysts
- Lymphatic malformations

Treatment

The treatment of choice for TGCs is complete removal of the cyst along with the extension to the back of the tongue. This is done in conjunction with removal of the central portion of the hyoid bone and is known as the Sistrunk procedure, named after the man who described it in 1920 (5). Delay in treatment often results in another infection which necessitates antibiotic therapy and delay of surgery until all the infection and inflammation are resolved (Fig. 3).

Removal is carried out under general anaesthesia. Approximately 10% of the cyst comes back and it is usually treated by a second removal. Recurrences are more common in patients who have had infected or previously drained TGCs [9,11,12].

Gioacchini et al. (2015) investigated 356 articles about TDGC and 24 were identified that satisfied selected criteria (a total of 1371 subjects) and concluded that a neck cyst mass is the main clinical presentation with a mean rate of 75%; that a most common complication after treatment is infection (4%) and that the mean overall recurrence was 11%. The Sistrunk procedure appears to be the better choice for the therapy of TGDCs to avoid recurrences. Further studies on larger cohorts of patients regarding the minimally invasive treatment options would be helpful to clarify and support their usefulness in selected cases [13].

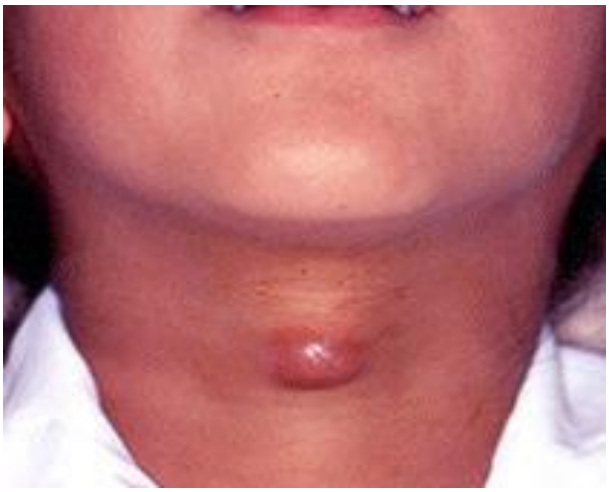

Fig. 3 TGC in 8 years old boy

Lendry et al. found that endoscopic removal with transoral laser microsurgery is a viable alternative to an external Sistrunk procedure in the case of an intralingual TGDC [14]

Huang et al. (2015) reported thirty-two patients from Beijing Tongren Hospital, Beijing, China, diagnosed with TGDCs who were selected. Seventeen patients with TGDCs were treated by traditional Sistrunk's surgery, and 15 patients underwent endoscopic cystectomy, and it was concluded that endoscope-assisted small-incision thyroglossal duct cystectomy is an efficient method. It causes smaller cosmetic defects and also reduces operative time. It will likely become the new standard procedure for patients with TGDCs [15].

Kim et al. stated that surgical treatment of midline TGDC via a retroauricular approach utilizing the robotic surgical system can be a technically doable and safe treatment option with outstanding cosmetic outcomes [16].

Many different operative methods have successfully treated recurrent thyroglossal duct remnants. To manage these challenging cases knowing the embryology, pathophisiology and applied anatomy is of paramount importance. Sistrunk procedure has the best cure rate.

Incomplete thyroglossal duct removal in the suprahyoid region mostly results in recurrences. The perihyoid, infrahyoid and tonque base are some other areas of recurrence. After a failed Sistrunk procedure, for management of recurrent disease, an extended or wide local incision is recommended: in the suprahyoid area including tongue base muscles and foramen cecum mucosa; removal of at least 2/3 of hyoid bone remnants, and a wide local incision of infrahyoid and the space posterior to the hyoid bone [17].

As mentioned above, many studies directed at the wider excision of the thyroglossal duct to completely excise the multiple and accessory tracts (“Christmas tree”) that are present in recurrent lesions and authors proposed novel techniques: repeat or extended [18] Sistrunk procedure, en bloc neck dissection, suture-guided transhyoid pharyngotomy, and Koempel's suprahyoid technique. Even though this review reports a 100% success rate with the 2 latter procedures, authors state that further prospective studies are required [18,19].

Cancer has been reported in a small number of patients in whom the cysts are not removed until adulthood [6]. Brewis et al. concluded that pediatric surgeons did fewer investigations than ENT surgeons and tended to excise more of the thyroglossal tract. Review of the published work suggests that ultrasound scanning and Sistrunk procedure are the best management policy [3,5] (Fig. 4).

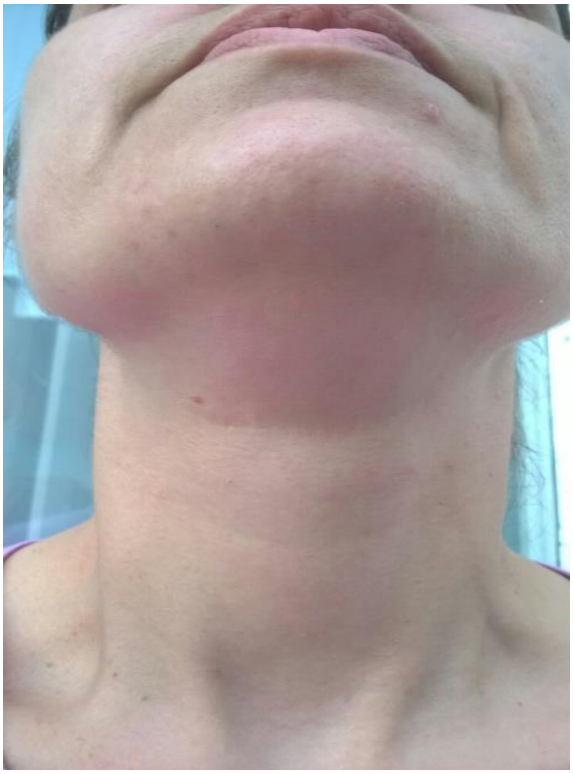

Fig. 4 Ten years after Sistrank procedure for TGC in a young female, without recurrence and complication. Good cosmetic appearance

Although rare, multiple recurrences have also been reported usually requiring wider removal of tissue in the region of the remaining hyoid bone. Recurrence is the most common complication of treatment and is managed by central neck dissection.

Complications

Infection is probably the most common complication.

Local growth and invasion are extremely uncommon.

Carcinoma is very rare and occurs in about 1-2% of patients.

Thyroid ectopia – fewer than 5% of these cysts actually have ectopic thyroid tissue.

Conclusion

Surgical treatment of choice for TGCs is Sistrunk operation which includes dissection of the hyoid bone to the base of the tongue.

Less than 5% of these cysts actually have ectopic thyroid tissue.

Cancer occurs approximately in 1–2 % of TGCs in patients in whom the cyst is not removed until adulthood.

Further studies are required to promote and establish novel treatment techniques, especially for recurrent cases.

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Case Report

DISSEMINATED INFECTION WITH BACILLUS CALMETTE-GUERIN AFTER BCG VACCINATION – CASE REPORT

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Abstract. *We report a case of disseminated tuberculosis caused by a vaccine strain of tuberculosis in a five-month-old infant that presented as severe systemic infection. Unfortunately, the infant died five days after admission. After the patient's death, the diagnosis was made based on pathohistological changes and a pharyngeal aspirate culture.*

Key words: *Calmette-Guerin Bacillus, tuberculosis, infant.*

Introduction

Tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis*, remains a leading public health problem worldwide [1]. In young children, tuberculosis is often disseminated due to the early, haematogenous spread of the bacterium after the primary pulmonary infection [2]. The first molecular evidence that a predisposition to tuberculosis might reflect inborn errors in immunity was provided by the occurrence of overwhelming tuberculosis in children with rare, severe primary immunodeficiencies (PIDs) [3, 4]. Disseminated disease in children with PIDs is often caused by widespread, weakly virulent mycobacteria, such as bacillus Calmette-Guerin (BCG) vaccines and environmental mycobacteria. There are some reports in the literature on patients with immunodeficiencies who developed tuberculosis [3, 4]. Complications of bacillus Calmette-Guerin (BCG) vaccination are uncommon. Fewer than one in 1000 vaccinated people develop significant local reactions, and serious disseminated disease develops in fewer than one in one million [5, 6].

Herein, we report a case of disseminated BCG tuberculosis caused by the bacillus Calmette-Guerin (BCG) vaccine in an infant who died of multiple organ failure.

Case Report

The patient was a boy that was born at term to nonconsanguineous parents of Roma origin. His birth size was normal, 50 cm and 3100 g, and he had no overt developmental defects. He was vaccinated with the BCG vaccine on the third day of life. Both parents, who had been previously vaccinated with BCG, were healthy, and he was their only child. The family's living conditions were good. The infant was healthy until 3.5 months of age, when fever and cough developed. He was hospitalised twice in a local hospital and received antibiotics due to urinary and respiratory infections. The treatment did not result in significant improvement, and the infections caused a failure to thrive.

When five months old, the infant was admitted to the intensive care unit of the Pediatric Clinic in Nis, Serbia, with fever, respiratory distress and lethargy. At the time of admission, he was severely ill and had a temperature of 39°C, a respiratory rate of 68 per minute and a heart of was 160 beats per minute. The patient was pale, underdeveloped and undernourished, and weighed 4400 g, which was below the 3rd percentile. The examination showed that he exhibited, nasal flaring, intercostal retractions, wheezing, decreased breath sounds over both lungs and granulomatous dermatitis. He was hypotonic, with modest spontaneous movements. The abdominal examination showed distension and hepatosplenomegaly.

Laboratory tests performed on admission revealed hypochromic anaemia and leukocytosis (table 1). Inflammatory parameters were high. The blood urea nitrogen, creatinine, and electrolyte levels were within normal limits. Arterial blood gas analysis showed mild respiratory alkalosis. Standard liver panel tests revealed the following results: ALT 29 IU/l (range 0–40); AST 69 U/L (range 0–40); alkaline phosphatase 346 U/L; total protein 49.4 gr/L; albumin 28 gr/L; total bilirubin 13.19 μmol/l; LDH 883 μ/l, uric acid 118 μmol/l; prothrombin time 11.5 seconds and partial prothrombin time 31.9 seconds. Immunological examination revealed

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Table 1 Laboratory findings

Variable	Reference range	At admission	4th day
Haematology			
Red blood cells ($\times 10^6/\mu L$)	4.70 – 5.80	4.08	4.06
Haemoglobin (g/dl)	11.0 – 18.0	7.8 ↓	8.7
Haematocrit (%)	51.0 – 59.0	28.8 ↓	30.7
Platelets ($\times 10^3/\mu L$)	100 – 310	200	251
White blood cells ($\times 10^3/\mu L$)	9.0 – 19.0	20.3	19.9
Neutrophils (%)	45.0 – 50.0	70.4	82.2
Monocytes (%)	3.0 – 5.0	3.0	2.4
Lymphocytes (%)	34.0 – 40.0	26.6	15.4
Biochemistry			
Total protein (g/L)	60 – 80	49.4 ↓	48.7
Albumin (g/L)	30 – 51	28 ↓	28.9
Parameters of inflammation			
C-reactive protein (mg/dl)	0 – 5	166	121
Fibrinogen (g/L)	2 – 4	6.79	/
Capillary blood gas analysis on FiO2 40%			
pH	7.35 – 7.45	7.52	7.54
PkO2 (mmHg)	60 – 80	39↓	48↓
PkCO2 (mmHg)	35 – 45	32 ↓	33↓
Virology, Bacteriology			
Elisa test for TORCH		Negative	/
Deep pharyngeal aspirate		*Escherichia coli*	/
Bacteriological culture		(ESBL+)	
Blood culture		Negative	/
Aspirate smear microscopy AFB		Negative	/
Mycobacterial culture		Positive: bacillus Calmette–Guérin	/
CSF analysis			
Haemorrhagic, Pandy +			
Biochemical analysis:			/
CSF glucose/serum glucose		1,5	
Cl (mEq/l)	96 – 120	120	
Cytological examination		Negative	/
Bacteriological culture		Negative	/
Immunological analysis			
IgA	0.058 – 0.858	<0.24	/
IgM (g/l)	0.264 – 1.46	0.20 ↓	/
IgG (g/l)	2.68 – 8.98	0.09 ↓	/
IgE (IU/ml)	< 15	<4.2	/

a decreased level of IgG 0.09 g/l and an IgM level of 0.20 g/l. Immunoglobulin A1 (IgA1) and IgE were within normal limits. The spontaneous and stimulated levels of reduction of nitroblue tetrazolium by neutrophilic polymorphonuclear leukocytes (NBT) were 6% and 68%, respectively. The results of the urine analysis was normal. The cerebrospinal fluid (CSF) obtained from the lumbar puncture was haemorrhagic. The blood culture was negative. A deep pharyngeal aspirate revealed *Escherichia coli* (ESBL+). The direct microscopic analysis of three deep pharyngeal aspirates for acid-fast bacilli was negative. Lowenstein Jensen culture of the deep pharyngeal aspirate identified bacillus Calmette-Guerin, but the results were not obtained until eight weeks after the patient's death.

A chest X-ray showed a slightly enlarged right hilar and perihilar and apical infiltrations (Figure 1). Abdominal sonography revealed mild hepatomegaly and prominent splenomegaly, with diffuse hypoechogenic focal changes (<10 mm) (Figure 2). CNS sonography revealed mild ventriculomegaly.

Initial therapy included fluids, oxygen, antibiotics (ceftazidime, vancomycin), and supplements, but the patent's symptoms did not resolve. The infant died due to multiple organ failure five days after admission.

Autopsy results showed granulomatous lesions in the spleen, liver (Figure 3) and skin. The pathohistological examination of the lungs showed changes typical of a cytomegalovirus infection.

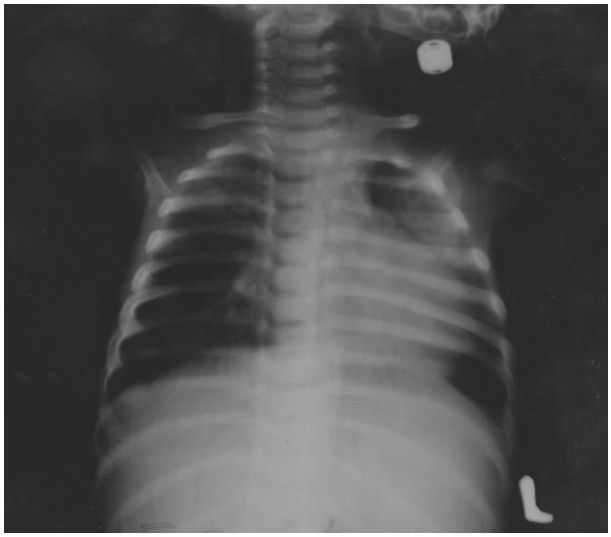

Fig. 1 Chest X-ray showed slightly enlarged right hilar lymph node and perihilar and apical infiltrations.

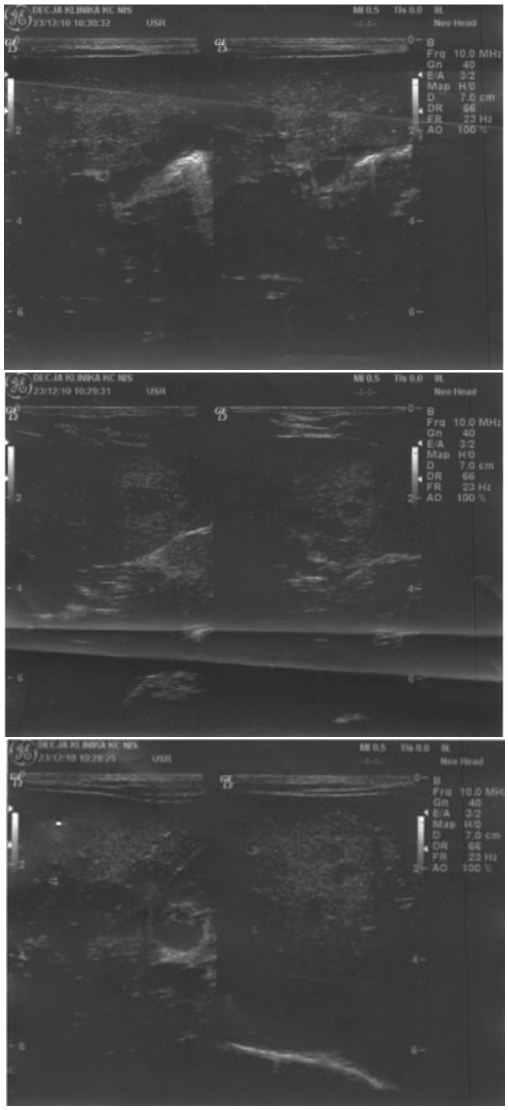

Fig 2 Mild hepatomegaly and prominent splenomegaly, with diffuse hipoechogenic focal changes (<10mm).

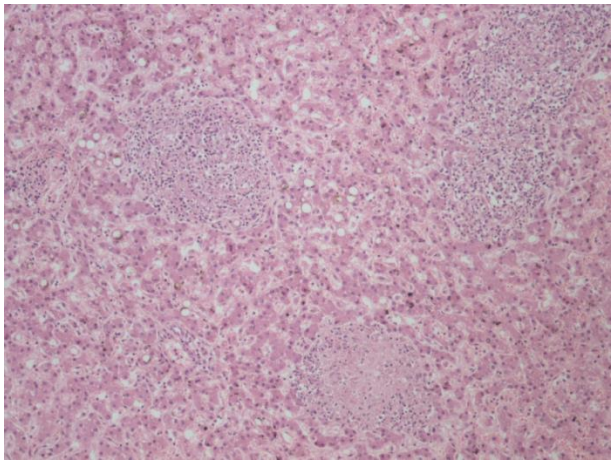

Fig. 3 There are granulomas (caseous) made of epithelioid cells in the liver parenchyma. There is caseous necrosis in the center of certain granulomas, and Langhan's giant cells (H&E 10) on the periphery.

Discussion

Tuberculosis (TB) continues to be one of the most devastating and widespread infections in the world. Young children are most likely to develop the disease after infection and are significantly more likely to develop extra-pulmonary and severely disseminated disease than adults [5].

Severe disseminated disease occurs early after the infection, within the first 2 to 6 months, and may represent an uncontrolled primary infection in children. The clinical manifestations of disseminated disease are protean, with involvement of the lungs, spleen, and bone marrow. Like adults with miliary TB, children are usually smear negative. With progressive pulmonary disease, respiratory distress, hypoxia, and pneumothorax/ pneumomediastinum may occur. Symptoms of the disease in our patients were fever, loss of appetite, respiratory distress and lethargy. The initial manifestation of disease in our patient involved the lungs, liver, spleen, and skin and included the possible early stages of meningitis.

Immunity to tuberculosis involves complex interactions between various cell populations to control and contain the infection and prevent further reactivation, but this response can also contribute to tissue damage. Cell-mediated immune mechanisms provide protection and delayed-type hypersensitivity [7]. The balance of Th1 and Th2 cytokines appears critical to controlling TB infection. Further evidence of the importance of interferon-γ can be found in children with hereditary IFN-γ receptor 1 deficiency. These children are prone to overwhelming infections with environmental mycobacteria and to the dissemination of bacilli Calmette-Guerin (BCG) after BCG vaccination. Together these data suggest that Th1 responses are protective, whereas Th2 responses are associated with chronic disease [8, 9]. In our patient, we ruled out chronic granulomatous disease using the NBT test. The serum levels of IgM and IgG were

decreased in comparison to the age-appropriate reference values. The initial treatment consisted of antibiotics, oxygenotherapy, and symptomatic therapy, that is, therapy to treat severe sepsis. Eight weeks after the patient's death, the Lowenstein Jensen culture identified bacillus Calmette-Guerin. We were not able to perform more detailed immunological tests. Cases of tuberculosis in children with malignant diseases [10, 11] and immunodeficiencies [12] have been reported in the literature, but there are few cases involving deficiencies in the receptors for interferon gamma and immunoregulatory factor 8 [8, 9, 13].

A specific immunodeficiency can be identified in only about half of the cases disseminated BCG infection [14]. In the other cases, the pathogenesis remains unclear. Such idiopathic cases have been reported in 24 countries, with a prevalence in France of at least 0.59 case per 1 million children vaccinated with BCG [15]. The high rates of consanguinity (30 per cent) and familial forms (17 per cent) and the equal sex distribution support the hypothesis of a new type of primary immune disorder with an autosomal recessive pattern of inheritance [15]. The parents of our patient are nonconsanguineous, and there were no similar diseases in other family members.

Conclusion

We have presented the case of an infant with disseminated mycobacterial disease after BCG vaccination that had a fatal outcome. Despite the use of modern diagnostics and treatments, such cases are difficult to diagnose and are even more difficult to cure. In most countries, the definitive diagnosis of macrophage function disorders (which involves analysing interleukin 12, interferon gamma, and the receptors for these cytokines and immunoregulatory factors) is difficult, which contribute to severe infections such as disseminated tuberculosis. To decrease the morbidity and mortality rates of disseminated tuberculosis caused by the BCG vaccine, further research is required to identify the basic causes of immune system disorders and to develop new therapies.

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