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TITLE OF THE DOCTORAL THESIS

**POST-MORTEM HISTOPATHOLOGICAL INVESTIGATION IN FORENSIC MEDICINE:**
**FOCUS ON BONE MARROW SAMPLING AND PROCESSING**

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**SOMMARIO**

**Introduzione.** Nel presente lavoro sono stati valutati una serie di campioni di midollo osseo provenienti da autopsie giudiziarie allo scopo di valutare le modificazioni morfologiche post-mortali e l'eventuale possibilità di utilizzare l'analisi immunofenotipica per scopi medico-legali.

**Materiali e metodi.** Sono stati esaminati 36 campioni di sterno prelevati in sede autoptica: 11 campioni sono stati presi da soggetti morti per sospetto annegamento nel periodo 2015-2017. Dopo il prelievo i campioni erano posti in contenitori di plastica e conservati refrigerati a (-20 C°) senza ulteriori trattamenti. Dieci campioni erano prelevati da soggetti morti per cause diverse; tali campioni erano fissati in formalina per un tempo variabile. 15 campioni erano prelevati da autopsie non giudiziarie. Su tutti i campioni era eseguita colorazione ematossilina-eosina dopo decalcificazione- fissazione. I dettagli morfologici erano valutati da tre differenti patologi. Parte di ciascun singolo sterno veniva decalcificata e fissati per tempi diversi in un *range* di giorni. Per la decalcificazione era utilizzato il decalcificatore in grado di decalcificare e fissare allo stesso tempo. Su tutti i campioni è stata eseguita l'analisi immunoistochimica utilizzando i seguenti anticorpi commerciali: CD20, CD3, CD71, CD61, mielo-perossidasi e CD34 di cui sono commercialmente disponibile i cloni. L'analisi dei preparati è stata eseguita utilizzando sia il microscopio Olympus BX51 sia il Fluo-scan-scope dopo digitalizzazione dei vetrini (e-slide).

**Risultati.** I campioni di midollo osseo sono risultati interpretabili morfologicamente in 21 dei 36 casi studiati (58%) e completamente interpretabili (dopo la valutazione morfologica e immunofenotipica) in 22 dei 36 casi esaminati (61%). Nello specifico i risultati sono stati interpretabili in 1/11 (9%) dei campioni congelati, in 8/10 (80%) dei casi giudiziari e in 13/15 (87%) dei casi non giudiziari. La presenza di cellule ematopoietiche tri-lineari (cellule mielo-eritroidi e megacariociti) era identificata sia a livello morfologico che immunofenotipico in tutti i gruppi ad eccezione della serie congelata dove si osservava tessuto degenerativo apoptotico. In uno solo dei campioni congelati il dato risultato risultava interpretabile; i caratteri di cellularità immunofenotipici così come la

distinzione della componente eritroide e mieloide era mantenuta. Il resto dei campioni congelati permetteva la visualizzazione della cellularità del midollo osseo senza distinzione delle singole componenti.
7 dei 10 campioni dei casi giudiziari risultavano interpretabili da un puto di vista istopatologico. La cellularità e le componenti cellulari (distinzione tra linea mielo-eritroide e megacariociti) era possibile mediante immunoistochimica in 8 dei 10 casi. L'analisi immunofenotipica poteva essere ben interpretata solo dopo decalcificazione lenta e fissazione in un *range* di 8-14 giorni.
I campion congelati non risultavano adeguati per la valutazione istopatologica e per la successiva analisi immunofenotipica a causa della diffusa assenza di tessuto idoneo.
L'anticorpo CD71 era valutabili come *dots* nei campioni congelati senza dettagli morfologici ed era valutabile come percentuali di cellule; lo stesso anticorpo nei casi non giudiziari e nei campioni giudiziari era facilmente contabile. Gli anticorpi CD3, CD20 e CD34 non mostravano alcuna positività nello sterno congelato mentre nei rimanenti campioni mostravano una immuno-espressione appropriata rispetto alle cellule specifiche (cellule immature linfoidi della serie T e B).
**Conclusioni**. L'analisi dei dati sperimentali ha evidenziato 3 punti principali: 1. il processo slow *day-by-day slow decalcifying* usualmente permette di ottenere un campione di midollo osseo dalle autopsie giudiziarie morfologicamente interpretabile; 2. l'analisi immunofenotipica può essere eseguita sul tessuto solo dopo decalcificazione lenta, usualmente dopo 8-14 giorni; 3. campioni congelati non sono idonei per l'analisi isto-patologica.
Tali dati portano a formulare la seguente valutazione conclusiva rispetto all'uso del midollo osseo in ambito forense. Il prelievo di sterno al fine di ottenere midollo osseo non è mandatorio in tutte le autopsie giudiziarie ma risulta raccomandabile nei casi di sospetta malattia ematologica, nei casi morte improvvisa, nei casi di morte su base settica. In questi casi si dovrebbe procedere al prelievo di una sezione di sterno di 2x3 cm; il campione dovrebbe essere sottoposto a fissazione e lenta decalcificazione per un tempo ottimale di 8-14 giorni. Mediante tale procedura si ottengono sezioni di midollo osseo morfologicamente interpretabili su cui è possibile eseguire analisi immunofenotipiche.

**ABSTRACT**

**Background**. We sought to investigate a series of bone marrow specimens from forensic autopsies, with focus on post-mortem morphological changes and utility of the immunophenotypical analysis for medical-legal purposes.

**Materials and Methods**. Thirty-six samples of the sternum taken during autopsies were examined: 11 samples were taken from subjects who died due to suspected drowning, in the period 2015-2017; the samples were placed inside a plastic container and stored in the freezer (-20C°) without being subjected to further treatments. 10 samples were taken from subjects who died due to several causes; the samples were fixed in formalin for a variable time; 15 samples were taken from non-forensic autopsies.

H&E staining has been performed on all samples after decalcification&fixation.

Morphological details has been categorized by three pathologists. Part of single sternum has been decalcified and fixed at different time (range in days). Decalcifier I is a unique decalcifying solution that has the ability to fix and decalcify at the same time.

Immunohistochemistry has been performed on all cases after inclusion of the following commercially available antibodies: CD20, CD3, CD71, CD61, myeloperoxidase and CD34 (commercially available clones). Data analysis has been performed by using both the Olympus BX51 microscope and D-Sight/Menarini Fluo-scanscope, after digitalization of the glass slides (e-slide).

**Results**. Overall, bone marrow samples were morphologically interpretable in 21/36 (58%) cases, were fully (after morphological and immunophenotypical data) interpretable in 22/36 (61%) cases, respectively 1/11 (9%) in the frozen samples, in 8/10 (80%) in the prospective *day-by-day slow* decalcified series and in 13/15 (87%) non-forensic series.

All trilinear haematopoietic cells (myelo-erythroid cells and megakaryocytes) were detailed at both morphology and immunophenotypical levels in all groups expect in the frozen series, where the apoptotic degenerative tissue was observed. In the single cases (1/11%, 9%) of samples taken at frozen temperature resulted interpretable; cellularity and immunophenotypical characters such as distinction in between myeloid and erythroid components were maintained. The rest of the frozen series

(9 cases out of 11, 91%) allowed visualization of the overall bone marrow cellularity, without distinction of the single components. In the prospective *day-by-day* decalcified series 7/10 (70%) of cases were interpretable for histopathology. Cellularity and detailed cellular components (mielo-erythroid distinction and megakaryocytes) by using immunohistochemistry were distincted in 8/10 (80%). Immunophenotypical analysis may be well interpreted on tissue after *slow* decalcification and fixation within a 8-14 range days. Frozen samples are not recommended for histopathological evaluation and subsequent immunophenotypical analysis, due to diffuse absence of suitable tissue.

CD71 was assessable as dots at frozen samples, without morphological details, and be evaluable as percentages of cells. CD71 in non-forensic and prospective cases was easily countable.

CD3, CD20 and CD34 did not show any positivity in the frozen sternum, whereas in the remaining series showed immunoexpression according to appropriate specific cells (T and B lymphoid and immature cells).

**Conclusions**. We found that: 1. slow *day-by-day* decalcifying process usually let the bone marrow from sternum after forensic autopsies, be morphologically interpretable; 2. immunophenotypical analysis may be performed on tissue after slow decalcification and fixation usually within 8-14 days; 3. frozen samples are not recommended for histopathological evaluation.

The above reported considerations lead to the following final conclusion. The sternum collection in forensic autopsies in order to evaluate the bone marrow is not mandatory in all the forensics cases. However it is recommended in all the cases of suspected haematological diseases, sudden deaths and septic deaths. In these cases the forensic pathologist must collect a section of the sternum 2x3 cm, which must be subjected to slow decalcification and fixation within 8-14 days. Following this procedure it is possible to obtain interpretable bone marrow preparations suitable also for the immunophenotypical analysis.

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## INTRODUCTION

Bone marrow samples is still not a routine procedure in the context of forensic autopsies, due to few data available.

We sought to investigate a series of sternum obtained from autopsies (forensic and non-forensics), to evaluate sensibility and specificity of detailed morphology and immunophenotyical findings in forensic cases.

## 1. BONE MARROW

### 1.1 ANATOMY AND PHYSIOLOGY

In the human embryo during the third week of development, groups of stem cells appear; some of these cells in the third month of embryogenesis migrate into the liver. This organ is the main site of blood cell formation until just before birth. Starting from the fourth month of gestation, the hematopoiesis begins also in the bone marrow. At birth, the bone marrow of the whole skeletal system is involved in the hematopoiesis until puberty.

From the age of 18 the hematopoietic bone marrow (red) is present only in the vertebrae, in the ribs, in the sternum, in the skull, in the pelvic bones, and in the proximal epiphyseal regions of the humerus and femur [Kuma V. et al., 2014] [figure 1].

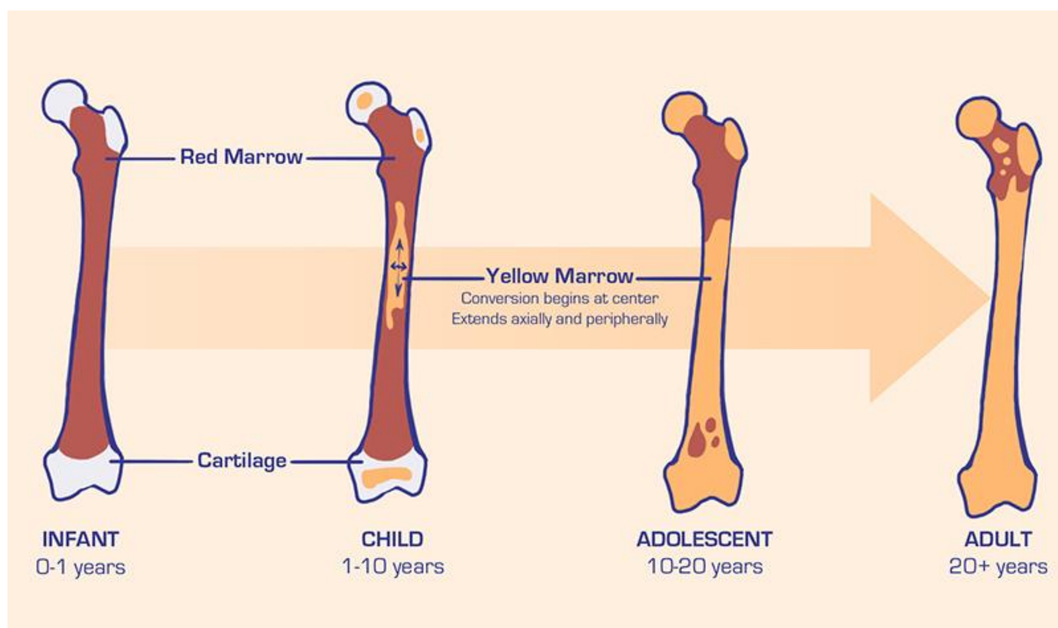


**Figure 1**: amount of red bone marrow-age of the subject.

The bone marrow is found within the central cavities of axial and long bones. The bone marrow has three components: hematopoietic cells, stroma/microenvironment, and medullary bone. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses interspersed within a meshwork of trabecular bone. The bone marrow is the major hematopoietic organ and a primary lymphoid tissue, responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes and platelets. The inner surface of the bone cavities and the outer surface of the cancellous bone spicules within the cavities are covered by an endosteal lining consisting of a single layer of flat “bone-lining cells” supported by a thin layer of reticular connective tissue; osteoblasts and osteoclasts are also found within the endosteal lining. In long bones, one or more nutrient canals (containing a nutrient artery and 1 or 2 nutrient veins) pass through the cortical bone entering the marrow cavity obliquely. In flat bones, the marrow is served by numerous blood vessels of various sizes entering the marrow via large and small nutrient canals [figure 2, 3].

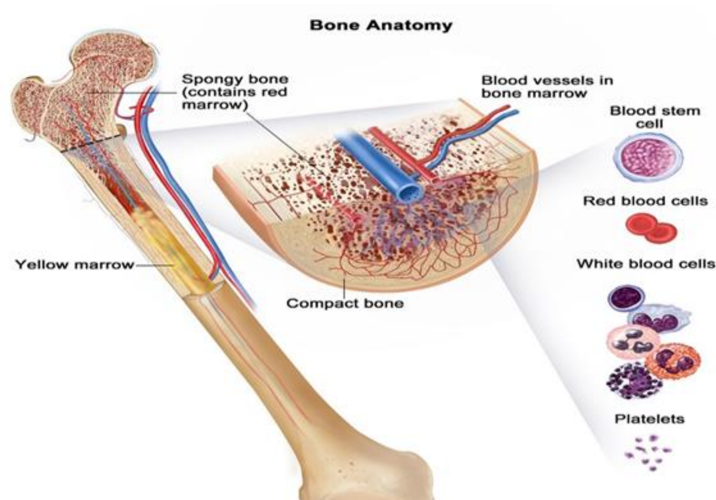


**Figure 2:** bone marrow structure.

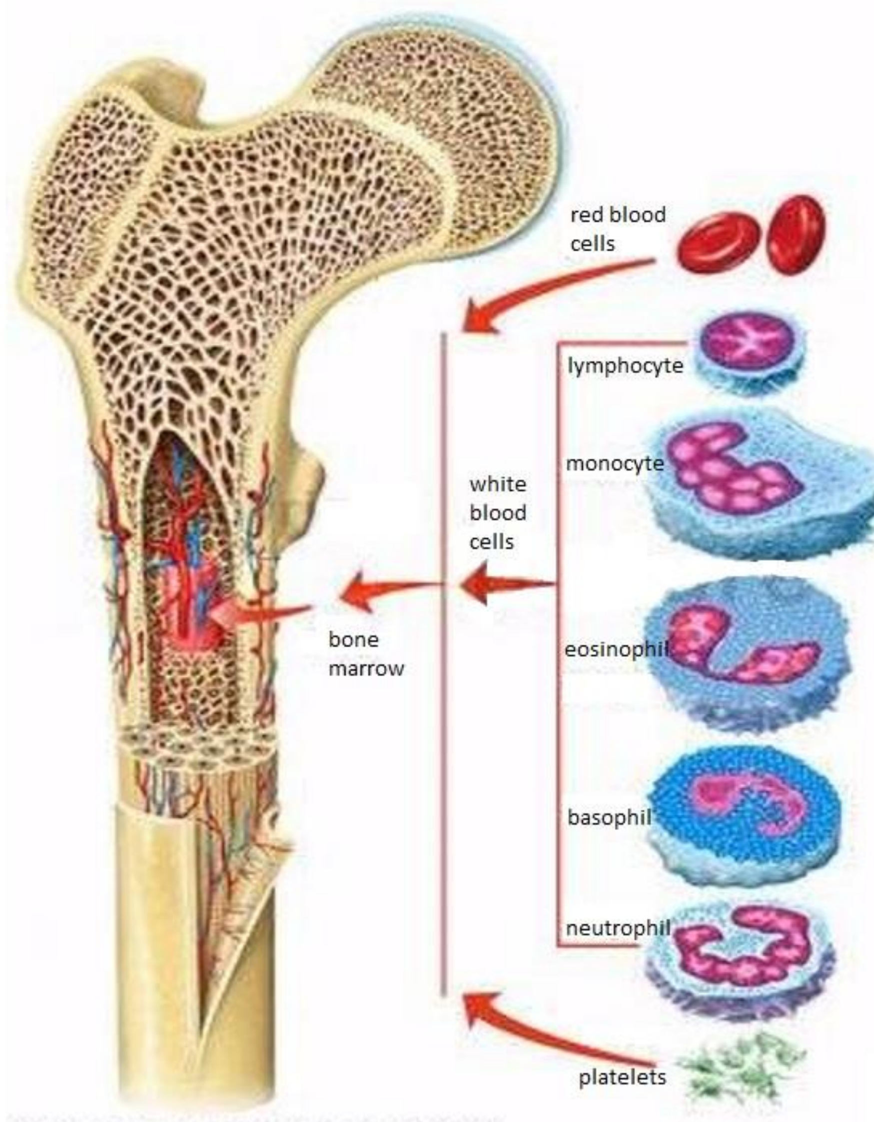


**Figure 3:** bone marrow structure.

After entry, the artery splits into ascending and descending branches that run parallel to the long axis in the central part of the marrow cavity, coiling around the primary venous marrow channel, the central longitudinal vein. These artery branches give rise to a multitude of small thin-walled arterioles and capillaries that extend outwardly toward the cortical bone. Near the bone, the arterioles open up and anastomose with a plexus of venous sinuses. These venous sinuses drain via collecting venules that lead back centrally to the central longitudinal vein that then drains via the nutrient veins. The marrow has an extensive blood supply. Also, it appears that nutrient artery-derived capillaries extend into the Haversian canals, return to the marrow cavity then open into the venous sinuses. Thus, there is a

circular pattern to blood flow within the marrow cavity, from the center of the marrow cavity toward the periphery of the marrow cavity then back toward the center. In long and flat bones, the blood supplies of the bone and bone marrow are interconnected through an endosteal network of vessels. The venous sinuses are thin-walled, consisting of a layer of flat endothelial cells with little to no basement membrane. The marrow does not have lymphatic drainage. The hematopoietic tissue consists of a variety of cell types including, the blood cells and their precursors, adventitial/barrier cells, adipocytes, and macrophages. The hematopoietic tissue cells are not randomly arranged but demonstrate a particular organization within the tissue [Travlos GS et al, 2006; Weiss L. et al, 1991][Figure 4].

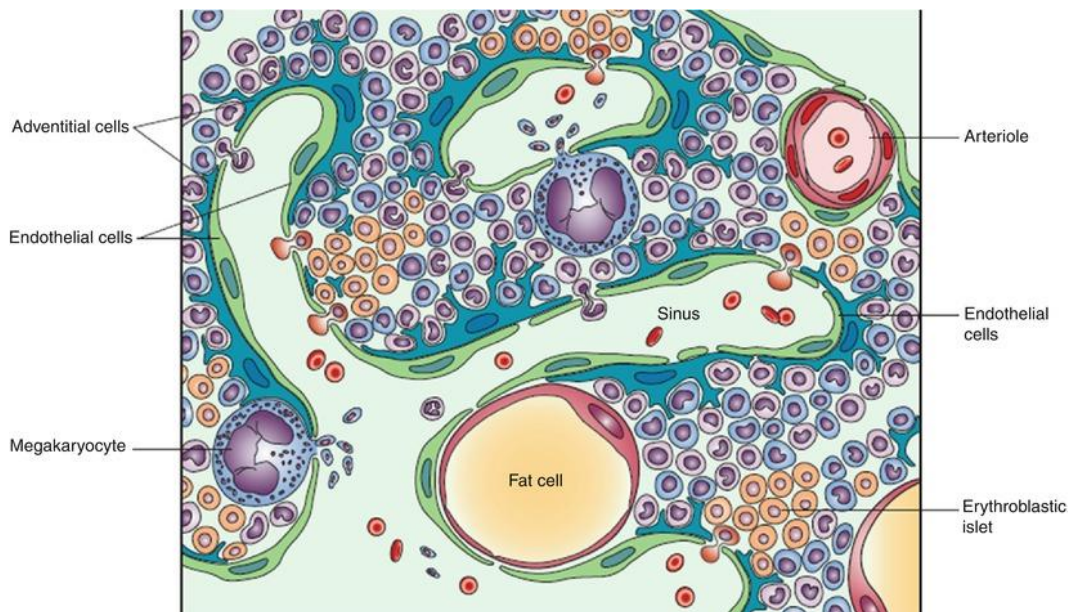


**Figure 4:** histology of the bone marrow.

For hematopoiesis to occur it must be supported by a microenvironment that is able to recognize and retain hematopoietic stem cells and provide the factors (e.g., cytokines) required to support proliferation, differentiation and maturation of stem cells along committed lineages.

The functional unit of hematopoiesis, the bone marrow microenvironment, includes the capillary-venus sinus, the surrounding extra-cellular matrix, and stromal cells (fibroblasts, macrophages, endothelial cells, adipocytes, and reticular cells). The

extracellular matrix includes regulatory factors as well as fibronectin, collagen, laminin, and various proteoglycans. Interactions with the bone marrow microenvironment determine the fate of hematopoietic stem cells, since all aspects of hematopoietic stem cell activity are influenced by bone marrow microenvironment niches [MaiKa M. et al, 2011; Foucar K. et al, 2008]

Upon maturation, the hematopoietic cells, regulated by the barrier cells, traverse the wall of the venous sinuses to enter the bloodstream; platelets are released directly into the blood from cytoplasmic processes of megakaryocytes penetrating through the sinus wall into the sinus lumen.

Some of humoral factors act on the more primitive cells and have a general action, while others act on later progenitors of a specific cell line. Most interleukins, B-cell growth factor, and B-cell differentiation factor are derived from T-lymphocytes. IL-1 is produced by macrophages.

Hematopoiesis is a continuous process, but can be separated into distinct stages. The first stage involves uncommitted (pluripotent) stem cells contained in the bone marrow. These pluripotent cells have two primary functions. First, they maintain their numbers by a process of self renewal and, secondly, they have the ability to give rise to all hematopoietic cells (erythrocytes, granulocytes, lymphocytes, monocytes, and platelets) [Travlos GS. Et al, 2006][Figure 5].

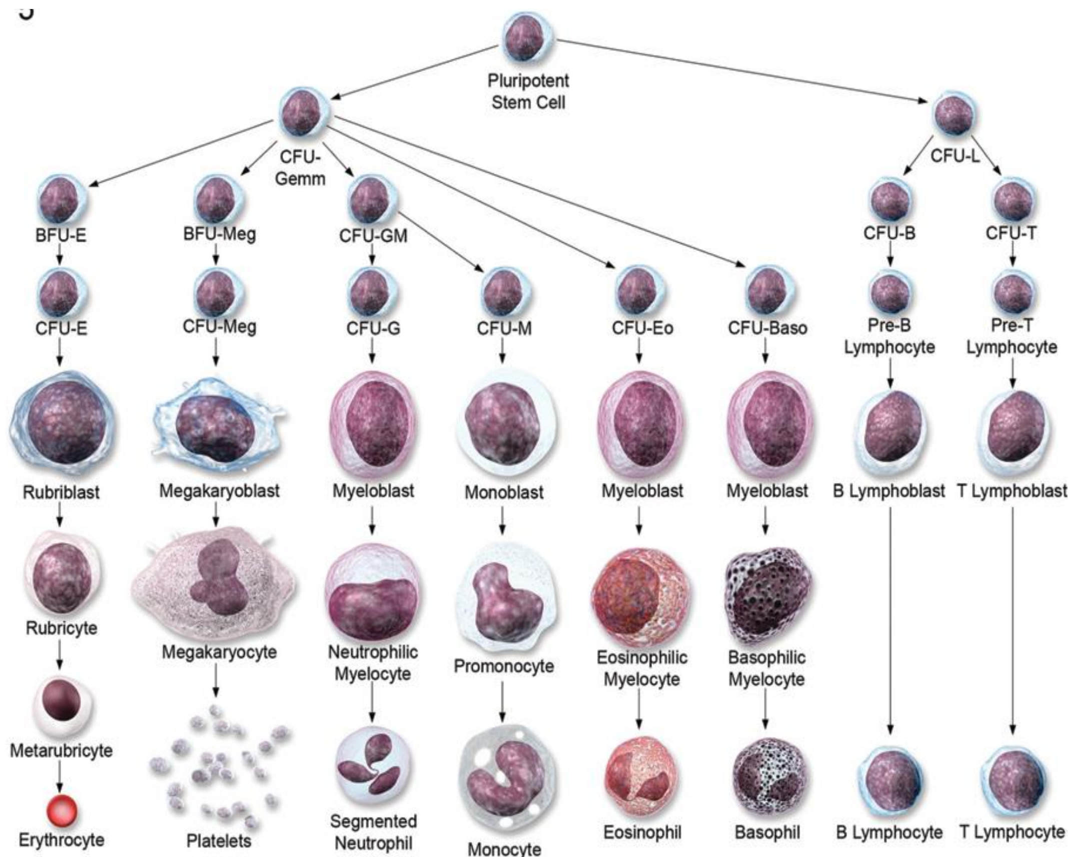


**Figure 5**: Representation of the maturation progression of the multiple cellular lineages present in the bone marrow. CFU = colony forming unit; E = erythyroid; Meg = megakaryocyte; Gemm = granulocytic, erythyroid, monocyte-macrophage, and megakaryocytic; GM = granulocyte/monocyte; G = granulocyte; M = monocyte; Eo = eosinophil; Baso = basophil; L = lymphocyte. Drawing by David Sabio.

A representative estimate of bone marrow activity can be obtained by examining the relationship between adipose cells and haematopoietic cells in bone marrow biopsies. In normal conditions, this ratio is about one, but in the condition of medullary hypoplasia the proportion of the adipocytes increases. The fat cells instead can also disappear in the conditions of increased haematopoiesis.

Normally, the bone marrow contains 60% of granulocytes and their precursors; 20% of erythroid precursors; 10% of lymphocytes and monocytes and their precursors and 10% of unidentified cells or in the process of destruction. As a result, the myeloid/erythroid ratio is 3: 1 [Orazi A. et al, 2007].

With the increase of the age of the subject or in particular pathological conditions the bone marrow can change its composition. Reactive marrow changes can be

quantitative (hyperplasia or hypoplasia) or qualitative (left-shifted maturation, cyologic atypia), and they can affect one or multiple hematopoietic lineages as well as the lymphoid, histiocytic, or stromal marrow compartments. Causes of reactive bone marrow changes typically originate outside of the marrow itself. The differential diagnosis includes autoimmune disease, nutritional deficiency or excess, toxic insults, medications, and infections [Tracy I. et al, 2018].

Hormones also play a physiological role. Hormones of the pituitary, adrenals, thyroid and gonads appear to participate in erythropoiesis by altering erythropoietin production and erythroid progenitor response to other factors. For example, androgens, thyroxine and growth hormone increase erythropoietin production; estrogen has an inhibitory erythropoietic effect. Hematopoietic tissue is also sensitive to external influences and can become suppressed in response to dietary restriction, chronic inflammation, and proliferative or neoplastic disorders [Travlos GS. et al, 2006].

With aging there is a progressive and physiological reduction of the amount of hemato-poietic bone marrow (red marrow) and an increase in adipose cells (yellow marrow). In fact, the marrow cellularity is estimated by the percentage of hemato-poietic cells in the total volume of marrow space; it declines with age, showing the highest cellularity in an infant or a young child and the lowest in an elderly person. Some examples are shown below.

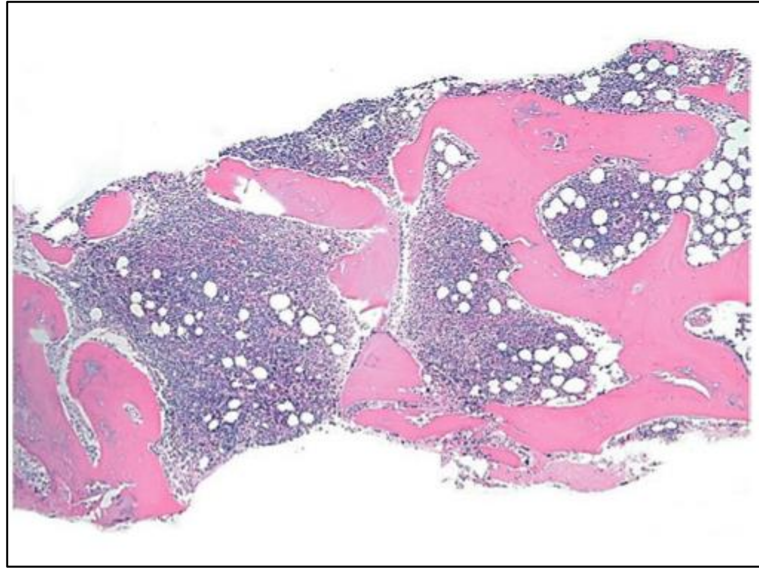

**Figure 6:** biopsy of 4 year-old boy is shown; the bone marrow space is occupied by approximately 90% cellularity with hematopoietic cells and approximately 10% by adipose cells, which is normal cellularity for the age of 4 years [Tracy I. eta al, 2018].

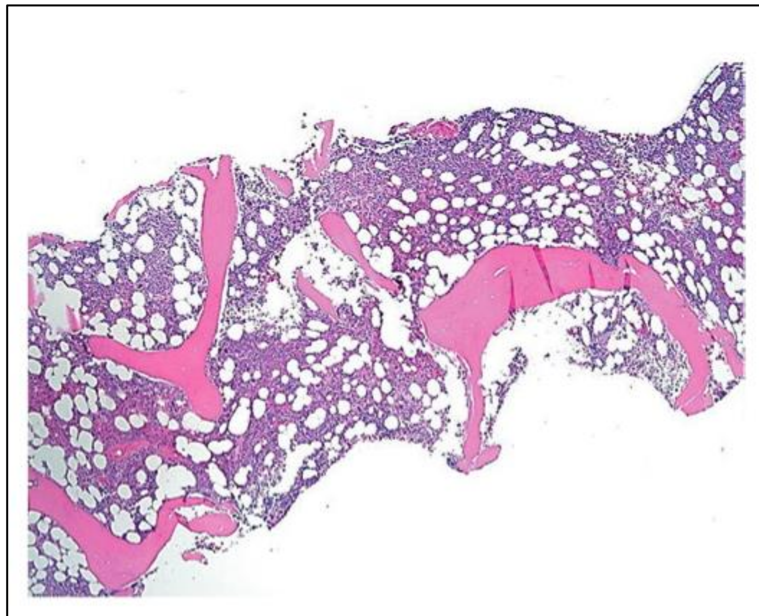

**Figure 7:** biopsy of 42 year old woman is shown; the marrow space is occupied by approximately 50-60% hematopoietic cells with trilineage hematopoiesis and approximately 40-50% adipose cells, which is normal cellularity for the age of 42 years [Tracy I. eta al, 2018].

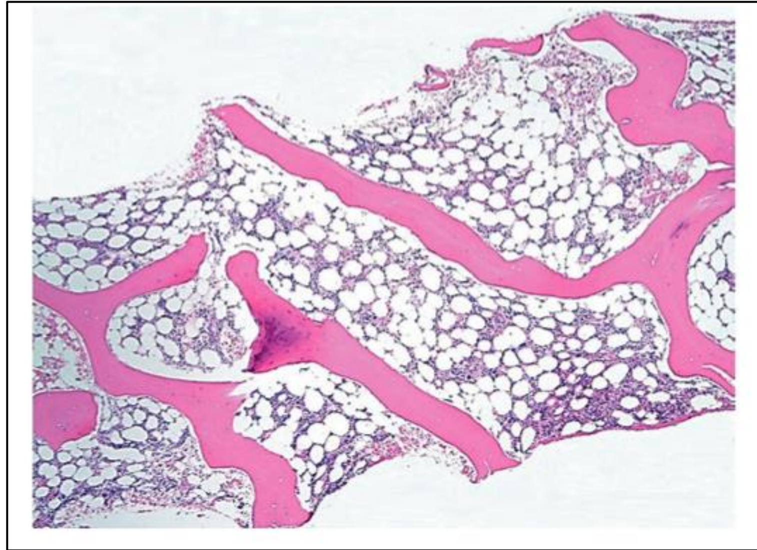

**Figure 8:** biopsy of 78 year old man is shown; the marrow space has significantly reduced hematopoietic cells (approximately 20%), which are replaced by adipose cells (approximately 80%). The cellularity in this figure is normal for the age of 78 years [Tracy I. eta al, 2018].

## 1.2 DIAGNOSTIC ASPECTS

In the clinical setting, the bone marrow examination is an important diagnostic procedure used for a wide variety of clinical conditions including the diagnosis of myeloid or lymphoid neoplasms, various reactive conditions or metastatic, non-hematopoietic malignancies. Bone marrow examination is also used for confirmation or monitoring of a remission state, residual or recurrent disease state, or regeneration of bone marrow after various therapies.

In any case, bone marrow analysis is a part of a multi-pronged evaluation of the hematopoietic system which includes also peripheral blood exam, complete blood count with differential, bone marrow smear exam and evaluation of cytocentrifuge preparations and/or bone marrow histopathology.

Bone marrow aspiration and biopsy are considered to represent overall bone marrow function [Tracy I. eta al, 2018].

Bone marrow samples can be obtained by:

- aspiration: a sample of the fluid part of the bone marrow containing the cells is

collected through the needle aspiration of the bone marrow so that they can be examined under a microscope. The sample is swiped on a slide and stained. The analysis evaluates the cell number, type, maturity, appearance, etc. [Figure 9];

**Figure 9**: aspiration of bone marrow on a slide.

- osteomidullary biopsy: a sample of bone marrow tissue is taken, preserving the structure of the tissue. A larger caliber needle is inserted into the bone and the instrument is withdrawn into which a small cylindrical fragment of tissue remains. The fragment can be fixed in formalin, decalcified, paraffin-embedded, sectioned and stained. [figure 10];

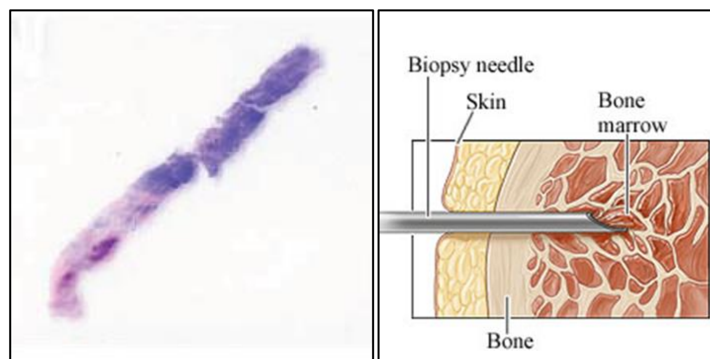


**Figure 10:** osteomidullary biopsy

Histopathology is a subjective assessment and is useful for evaluating bone marrow architecture, assessment of cellularity, estimation of M:E ratio (limited sensitivity), assessment of cell lineages, estimation of iron stores and other features (e.g., neoplasia, inflammation, pigment, infectious agents).

In general, decalcified, paraffin-embedded, haematoxylin and eosin (H&E)-stained sections of bone marrow, the more mature stages of the erythroid and myeloid cells, adipocytes, mast cells, and megakaryocytes can be identified, but stem cells,

immature myeloid, erythroid, lymphoid, monocytoid and stromal cells cannot be identified consistently. An estimation of general hematopoietic activity and the myeloid/erythroid ratio can also be performed [Travlos GS et al, 2006].

For histopathology, numerous variables regarding specimen collection and processing (e.g., fixation, decalcification, embedding, sectioning and staining) may affect the quality of the sample to be evaluated and must be considered.

*Fixation*

After the withdrawal, marrow should be collected as soon as possible for histological assessment. Fixative solutions stabilize tissue by cross-linking proteins; the degree of cross-linking may result in denaturation of proteins and affects morphological, cytochemical or immunohistochemical qualities of the specimen.

Because its readily available, 10% neutral buffered formalin (10% NBF) is the most commonly used fixative. For routine H&E sections, no special handling is required prior to tissue processing. 10% NBF-fixed tissues may be used for frozen sections and evaluation of adipocytes.

For specimens to be used for immunohistochemistry, a prolonged fixation in 10% NBF may be detrimental (e.g., loss of antigenic epitopes due to continued amino group crosslinking and alteration of tertiary protein structure). Transferring the tissue to 70% ethanol after a 12-hour fixation reduces fixation-related effects.

*Decalcification*

For paraffin-embedded sections, bone marrow specimens must be decalcified prior to sectioning; specimens for plastic embedding do not. There are numerous types of decalcifiers (e.g., acids, chelators, resins) and methods (e.g., immersion, sonication, microwave, ion-exchange) for the decalcification of bone marrow specimens. If preservation of enzyme reactivity or antigenic sites is required, selection of the method of decalcification is important. With larger specimens, some damage to tissue morphology, due to decalcification, is almost unavoidable (especially with the use of acid decalcifiers).

Both organic and mineral acids are commonly used for decalcification of bone marrow specimens. Organic acids (e.g., formic and acetic) decalcify slower than mineral acids (e.g., HCL and nitric). Mineral acids are commonly used in the rapid-type decalcification methods. Over decalcification of tissue with acids, particularly

mineral acids, results in tissue destruction. Thus, tissue in acid decalcifiers should not be left unchecked for extended periods (e.g., over a weekend). For proper decalcification, there must be even distribution of acid around bone sample. Thus, tissue suspension or mild agitation, such as gentle mixing (e.g., shaker/rocker) or air bubble percolation, may be useful. Additionally, acid decalcifiers may need frequent solution changes (e.g., daily) for adequate decalcification. In general, acid decalcification is not recommended for samples needed for enzyme or immunostaining. This caveat is particularly appropriate for the mineral acids like HCL or nitric; the organic acids appear to be somewhat better. All acid decalcifiers, especially mineral acids, reduce morphological quality and Giemsa stains may be unacceptable due to loss of basophilic-staining structures [Travlos GS et al, 2006]

In this regard Sung-Eun Choi *et al*, formulated a protocol that compared various decalcification methods (hydrochloric acid –HCl; ethylenediaminetetraacetic acid disodium salt dehydrate -EDTA; RDO-GOLD RDO), concluding that the EDTA protocol would be the best to preserve the genetic material. RDO can be an acceptable alternative when rapid decalcification is required [Sung EC. Et al, 2015]. Other authors suggest that decalcification is best achieved by 14% EDTA that sequesters metallic ions, including calcium, in aqueous solutions as a chelating factor for 16–24 h [Torlakovic EE. Et al, 2015]

However, new decalcifying solutions are being developed, and some may be less deleterious to bone marrow tissue even after short fixation. After fixation and decalcification, the tissue needs to be further processed before embedding in paraffin [Peterson LC. et al, 2001]

The most important problem is inadequate tissue dehydration and clearing prior to paraffin embedding. This can be prevented by preparing all solutions freshly every week, depending on the volume of tissue processed [Werner M. et al, 2000].

*Embedding*

The preparation of bone marrow sections in a laboratory of routine histology requires, after decalcification, the inclusion in paraffin. This technique has the disadvantage of producing a narrowing and a considerable loss of cellular detail. In the mid-1980s a new inclusion technique was evaluated using plastic inclusion. This work [Islam A, Frisch B.-1985] describes a simplified routine procedure for the use

of methyl methacrylate as a plastic embedding medium for the preparation of semi-thin sections of non-calcified bone marrow. The study also proposed a modification of the May-Grunwald-Giemsa stain that provides good color differentiation of various hematopoietic cells in the bone marrow. The authors concluded that the method was simple, reproducible, did not require expensive equipment and was suitable for routine processing of bone marrow biopsy nuclei in any histopathology laboratory [Islam A. et al, 1985].

However, other authors suggested that under optimal circumstances, morphology looks superior in plastic-embedded tissue. However, there is experiential evidence of excellent morphology with appropriate fixation time and thin sectioning (2 um) of tissue for formalin, AZF fixatives, and the need for embedding in plastic was challenged in 1987 by *Gatter et al*. Plastic embedding techniques may obviate the need for the decalcification step and thus eliminate one of the major variables impacting pre-analytical bone marrow IHC quality. However, only a few centres still cultivate expertise required for plastic embedding. Without a resurgence of interest and developmental efforts leading to broader use of plastic embedding, paraffin embedding will remain the method of choice in the great majority of laboratories. If plastic embedding could be automated and cost-effective, it could become a preferred choice for embedding of all types of tissue biopsies, not only bone marrow samples [Peterson G. et al, 2001].

For example *Krenacs T et al,* suggested the processing of bone marrow biopsies by buffered fixation of formaldehyde and inclusion of epoxy, with or without decalcification of EDTA. The technique can be considered as a period of time similar to that of paraffin processing with only a cost increase of about 30%, offering an alternative possibility to paraffin processing for diagnosis and research [Roll P. et al, 2009].

*Tissue Processing and Staining*

H&E-stained sections of paraffin-embedded bone marrow tissue are typically evaluated histologically. However, H&E does not provide always consistent hematopoietic cell differentiation. Section thickness is an important factor and 3-micron sections provide better cellular morphological detail compared to thicker (≥ 5-micron) sections. Giemsa-stained sections provides better morphological detail

compared to H&E-stained sections. For the Giemsa stain, however, acid-decalcified tissue results in loss of basophilic-staining structures. The use of chelating-type decalcifiers improves Giemsa staining. Plastic embedding provides a significant improvement in cellular morphology. Additionally, there is no need for decalcification, thus, reducing shrinkage artifact and loss of cellular detail related to decalcification methods. Thin sections (≤3 microns) can be easily produced, improving cytological quality. However, plastic embedding is time, labor and cost intensive and, thus, not justified for a high-throughput operation. Prussian Blue stain is used for the assessment of iron stores; acids in decalcifiers or fixatives may washout iron stores. Thus, tissue iron could be underestimate [Travlos GS. Et al, 2006].

## 1.3 BONE MARROW FEATURES OF SYSTEMIC INFECTIONS

For many systemic infections, the bone marrow findings are non-specific but compatible with a reactive process. Even when non-specific, the morphological evaluation of the bone marrow integrated with clinical history serves to further direct workup, including the need for special stains, cultures, serologies, or molecular studies.

Bone marrow changes in infectious and systemic diseases can be studied by morphology. Morphologically, similar lesions can arise from different pathological agents and one disease can cause several different lesions. Some aetiological agents can be detected by tissue sections that have been specially stained, and/or by immunohistochemistry. Malignant diseases can be associated with reactive changes. Two types of changes that result from the development of an inflammatory response or immune disorders can be seen. One lesion is characterized by stromal reactions in the connective tissue and microvascular bed, the second by the response of haematopoietic stem cells to local or general stimulation [Diebold J. et al, 2000]

***Acute inflammation***

Acute inflammation can be seen in a large number of infections, particularly in septicaemia; the manifestations of an acute inflammation can be of different types: essudative type; haemorrhagic type; necrotic type; suppurative type.

In the essudative type, interstitial oedema and small haemorrhages are associated

with arteriolo-capillary congestion. The endothelial cells are often hypertrophic. Sometimes stellate fibrin deposits are present between the haematopoietic cells. Intravascular coagulation may also be seen, particularly in severe infections, and this is often associated with haemorrhage or necrosis. Oedema should be distinguished from gelatinous transformation of bone marrow. This lesion is characterized by hyaline deposits between adipocytes in areas without any haematopoietic cells. Gelatinous transformation is observed in patients with severe malnutrition and protein loss and in HIV-positive patients [Diebold J. et al, 2000].
In the haemorrhagic type, the haemorrhages are often associated with hypoplasia of the haematopoietic cells.
Such alterations have been described in aplasia after hepatitis B infection or in patients with severe macrophage (histiocyte) activation syndrome.
In necrotic type, the ischaemic necrosis during the acute phase of inflammation is secondary to arteriolar thrombosis and may be found in patients with septicaemia, bacterial endocarditis, different bacterial, viral, fungal or parasitic infections, Q fever, dysimmune disorders such as disseminated erythematous lupus, or hypersensitivity. The progression of the necrosis is characterized by either a total or partial restitution. In some cases, collagenous fibrosis replaces the necrotic areas.
A particular type of necrosis occurs during the antiphospholipid syndrome and in tuberculosis. In acute tuberculosis with haematogenous diffusion, areas of caseous necrosis can be observed surrounded by a few histiocytes without epithelioid cells. Such caseous necrotic areas are present along the bone trabeculae or in the adipose tissue which replaces the haematopoietic cell clusters. Several the acid-resistant bacilli are easily observed in these necrotic areas through the use of Ziehl ± Neelsen staining.
The suppurative type lesions are only seen in acute osteomyelitis. During the acute phase, typical lesions are characterized by areas of suppurative necrosis containing basophilic cellular debris and neutrophils, with a variable number of macrophages.

***Chronic inflammation and immune reactions***

Stromal reactions also occur as a result of chronic inflammation. The most frequent lesion is characterized by the development of granulomas. However, lymphoid

hyperplasia and reactive plasmacytosis are also often observed, mainly because of deregulated immune reactions.

In the granulomatous chronic inflammation, granulomas are identified by an accumulation of histiocytes or epithelioid cells organized in a nodular pattern with sharp outlines. Giant cells may also be present. The centres of the granulomas may be occupied by different types of necroses according to the aetiology; for example, fibrinoid in hypersensitivity, caseous in tuberculosis. The granulomas may be surrounded by lymphocytes, with a predominance of T-cells over B-cells.

- Bacterial infections
  - Tuberculosis
  - Typhoid fever
  - Brucellosis
  - Leprosy
  - Syphilis
  - Legionnaire's disease
- Viral infections
  - EBV infections
  - Herpes zoster
  - HIV infections
- Rickettsial infection
  - Q fever
- Parasitic infections
  - Leishmaniosis
  - Toxoplasmosis
- Fungal infections
  - Histoplasmosis
  - Cryptococcosis
- Dysimmune disorders
  - Sarcoidosis
  - Drug hypersensitivity
- Malignant diseases (with/without tumourous bone involvement)
  - Hodgkin's disease
  - Malignant lymphoma of T- or B-cells
  - Multiple myeloma
  - Some carcinomas

**Figure 11**: Conditions associated with bone marrow granulomas [Brunning RD et al, 1994].

In the reactive lymphoid lesions, lymphoid cell hyperplasia has been described in numerous different diseases; lesions are more frequently seen in patients older than

50 years of age and in women. Thus there is not a clear distinction between lymphoid cell hyperplasia as a pathological or a physiological phenomenon. In younger patients, such lymphoid hyperplasias seem to be more often associated with various chronic inflammatorydiseases or immune disorders.

Histopathology and immunohistochemistry allow three different types of reactive lymphoid lesions to be distinguished:

-reactive lymphocyte aggregates;

-lymphoid follicles with germinal centres;

-reactive lymphohistiocytic infiltrates.

The most important differential diagnosis is represented by bone marrow involvement in malignant lymphomas. Reactive lymphoid aggregates should not be confused with early infiltrates of small B- or T-cell ML (B-CLL, lymphoplasmacytic ML, splenic marginal zone lymphoma, mantle cell lymphoma, T prolymphocytic leukaemia, etc.).

| Benign lymphoid lesions | Malignant lymphomas |
|---|---|
| No paratrabecular localization | Frequent paratrabecular localization |
| Often are well-circumscribed, roundish | Often have diffuse borders, irregular shape |
| No interstitial infiltrate between the lesions | Frequent infiltration into the adjacent marrow |
| Polymorphic cell population | Monomorphic cell population |
| Organized around a vessel | No vessel or vascular hyperplasia |
| No myelofibrosis | Often myelofibrosis in the lymphoma infiltrate |
| Sometimes presence of a reactive germinal centre | No reactive germinal centre |
| No large cells in the lesions or in the sinuses | Presence of large B- or T-cells, of lymphoid cells in the sinuses, of Reed–Sternberg cells |
| Absence of lymphoma cells on sternal puncture smears | Presence of lymphoma cells on sternal puncture smears |

**Figure 12**: Criteria for benign lymphoid lesions and malignant lymphomas including Hodgkin's disease [Brunning RD et al, 1994].

The immunoblastic hyperplasia is a very rare condition, never seen in isolation. In viral diseases, particularly in infectious mononucleosis, a small number of immunoblasts can be observed associated with reactive lymphoid lesions, mainly with reactive lymphohistiocytic infiltrates. They can be of the B-cell and/or T-cell type.

Plasmacytosis is a plasma cell hyperplasia which is commonly found in patients with chronic inflammatory diseases, in dysimmune disorders, in septicaemia, and in HIV infection.

Concerning histiocytic hyperplasia includes different types: histiocytosis with haemosiderin, disseminated histiocytosis, histiocytosis with haemophagocytic syndrome, histiocytosis with phagocytosis of infectious agents.

In the chronic inflammation with fibrosis, collagen bands can develop around vessels and/or granulomas. Areas of fibrosis can also replace previous necrosis. Interstitial oedema with a variable number of plasma and lymphoid cells may be associated.

In chronic diseases with amyloidosis, amyloid deposits may occur in chronic inflammations or in dysimmune diseases. The deposits are mainly present on the vessel walls or in the interstitium.

***Haematopoietic cell line modifications***

These modifications are probably secondary to the production of various cytokines which either block or stimulate the proliferation of haematopoietic stem cells (growth factors) and their differentiation.

In acute inflammation, an increase in the number of cells of the granulocytic series is often observed. In some cases, hyperplasia is associated with an accumulation of mature neutrophil granulocytes in the centre of the medullary spaces around the venous sinuses. In other cases, due to the rapid delivery of mature granulocytes to the peripheral blood through the venous sinuses, the majority of the cells of the granulocytic series are immature. In very severe infections (septicaemia), an absence of mature granulocytes is associated with a proliferation of myelocytes and promyelocytes, mimicking the process of acute or chronic leukaemia (leukaemoid reaction). An increase in megakaryocytes has been seen during infection, particularly in cases of disseminated intravascular coagulation. In different inflammatory diseases, anaemia is common and is responsible for an erythroblastic hyperplasia. In cases of severe anaemia, the accumulation of large basophilic erythroblasts may mimic myelodysplasia. In septicaemia, modifications of the three main cell lines can occur and may be responsible for false diagnosis of acute or chronic leukaemia or myelodysplasia. In severe infection, hypoplasia or even

aplasia can be observed, as well as oedema, haemorrhage, fibrin deposits, necrosis or even fibrotic organization.

In chronic inflammation, stimulation of stem cells leads to hyperplasia of one, two or all of the bone marrow haematopoietic normal cell lines. In some cases hyperplasia predominates among cells of the granulocytic series, with numerous promyelocytes and myelocytes along the bone trabeculae and mature neutrophil granulocytes in the centre of the medullary spaces. The number of eosinophil granulocytes can also be increased, particularly in cases of drug hypersensitivity, allergy, collagen diseases, inflammation due to parasites or Hodgkin and T-cell ML. In eosinophilic hyperplasia, macrophages may contain Charcot-Leyden crystals. Erythroblastic hyperplasia can be observed during infection with Parvovirus B19. Giant erythroblasts and multinucleated erythroblasts are dispersed in the bone marrow. Viral inclusions can be seen in the nuclei of erythroblasts. The other cell lines are normal. This viral infection of the erythroblasts can cause a severe anaemia or an anaplastic crisis.

# 2. EXPERIMENTAL PART

## 2.1 Aim of the Study

We sought to investigate a series of bone marrow specimens from forensic autopsies, with focus on postmortem morphological changes and utility of the immunophenotypical analysis for medical-legal purposes.

## 2.2 Material and Methods

### *Sampling*

Overall, thirty-six (three series) samples of the sternum taken during autopsies were examined:

**1)** 11 samples were taken from subjects who died due to suspected drowning, in the period 2015-2017. The samples were placed inside a plastic container and stored in the freezer (-20C°) without being subjected to further treatment (case 1-11);

***Case 1 (MY)***

Age: 35 years old;

Date of disappearance: 03.00 p.m of 02.08.2016;

Date of discovery: 05.30 p.m of 02.08.2016;

Date of the autopsy examination: 10.00 a.m. of 05.08.2016;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: BAC 0,17 g/dL.

***Case 2 (SJ)***

Age: 7 years old;

Date of disappearance: 04.00 p.m of 11.05.2016;

Date of discovery: 01.30 a.m of 12.05.2016;

Date of the autopsy examination: 11.00 a.m. of 14.05.2016;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: negative.

***Case 3 (BP)***

Age: 87 years old;

Date of disappearance: 01.00 p.m of 03.08.2016;

Date of discovery: 03.30 p.m of 03.08.2016;

Date of the autopsy examination: 11.00 a.m. of 06.08.2016;

Cause of death: acute myocardial event with ischemic or arrhythmic origin, in subjects with severe coronary artery disease and fibro-adipose substitution of myocardial tissue;

Toxicological data: BAC 0,29 g/dL.

***Case 4 (BF)***

Date of disappearance: 09.07.2016;

Date of discovery: 01.00 p.m of 21.10.2016;

Date of the autopsy examination: 11.00 a.m. of 27.10.2016;

Tanatological data: advanced state of putrefaction.

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water.

***Case 5 (TA)***

Age: 34 years old;

Date of disappearance: 05.00 p.m of 16.04.2017;

Date of discovery: 24.04.2017;

Date of the autopsy examination: 11.00 a.m. of 28.04.2017;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: BAC 0,9 g/dL.

***Case 6 (BL)***

Age: 52 years old;

Date of disappearance: 08.11.2017;

Date of discovery: 02.00 p.m of 09.11.2017;

Date of the autopsy examination: 11.00 a.m. of 10.11.2017;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: negative.

***Case 7 (RMG)***

Age: 83 years old;

Date of disappearance: 09.00 a.m of 22.10.2017;

Date of discovery: 12.00 a.m of 22.10.2017;

Date of the autopsy examination: 11.00 a.m. of 27.10.2017;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: negative.

***Case 8 (AG)***

Age: 79 years old;

Date of disappearance/Date of discovery: 10:15 a.m. of 22.06.2016;

Date of the autopsy examination: 11.00 a.m. of 24.06.2016;

Cause of death: cardiac death;

Toxicological data: 408 ng/ml of 7-amino-clonazepam

***Case 9 (HCD)***

Age: 48 years old;

Date of disappearance: 02.03.2015;

Date of discovery: 04.03.2015;

Date of the autopsy examination: 06.03.2015;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: negative.

***Case 10 (KV)***

Age: 31 years old;

Date of disappearance: 2-5 days prior to the discovery of the body;

Date of discovery: 24.11.2015;

Date of the autopsy examination: 11.00 a.m. of 25.11.2015;

Cause of death: acute respiratory failure secondary to mechanical asphyxiation from drowning in fresh water;

Toxicological data: BAC of 1,47 g/dL.

***Case 11 (DN)***

Age: 26 years old;

Date accident: 30.07.2016;

Date of death: 04.08.2016 at the hospital;

Date of the autopsy examination: 11.00 a.m. of 05.08.2016;

Cause of death: drowning syndrome;

Toxicological data: negative.

**2)** 10 samples were taken from subjects who died due to several causes; the samples were fixed in formalin for a variable time (case 12-21).

| Case number | Age (years) | Cause of death |
|---|---|---|
| 12 | 20 | post-anoxic encephalopathy |
| 13 | 64 | violent mechanical asphyxia |
| 14 | 48 | violent mechanical asphyxia |
| 15 | 44 | encephalic injury secondary to firearm injury |
| 16 | 64 | violent mechanical asphyxia |
| 17 | 48 | cardiac death in a drug addict |
| 18 | 48 | sepsis |
| 19 | 1 hour | respiratory failure |
| 20 | 6 month | myocarditis |
| 21 | 47 | polytrauma |

**3)** 15 samples taken from non-forensic autopsies (cases 22-36).

10 patients died for oncological advanced staged carcinomas and 5 for non-oncological causes such as failure of organ-solid transplantations (n. 3 patients) or critical multi-comorbidities in older ages (1 cases with liver diseases, 2 cases with interstitial pneumopatiae).

### *Histological and Immunohistochemical analysis*

#### *Haematoxylin and eosin staining*

H&E staining has been performed on all samples after decalcification&fixation.
Morphological details has been categorized by three pathologists. Part of single sternum has been decalcified and fixed at different time (range in days).
Decalcifier I is a unique decalcifying solution that has the ability to fix and decalcify at the same time. Bone specimens may be completely fixed and decalcified in approximately 2-4 hours or slow processing time (range in days) in order to preserve hemopoietic cells. Tissues decalcified in this solution cut with ease and routine H&E techniques produce excellent stained sections.

#### *Immunopanel of antibodies*

Immunohistochemistry has been performed on all cases after inclusion of the following commercially available antibodies: CD20, CD3, CD71, CD61, myeloperoxidase and CD34 (commercially available clones).

#### *Digital Pathology Interpretation*

Data analysis has been performed by using both the Olympus BX51 microscope and D-Sight/Menarini Fluo scan-scope, after digitalization of the glass slides (e-slide).

## 2.3 RESULTS

### Histopathological and immunophenotypical findings

Overall, bone marrow samples were morphologically interpretable in 21/36 (58%) cases, were fully (after morphological and immunophenotypical data) interpretable in 22/36 (61%) cases, respectively 1/11 (9%) in the frozen samples, in 8/10 (80%) in the prospective *day-by-day slow* decalcified series and in 13/15 (87%) non-forensic series.

All trilinear haematopoietic cells (myelo-eritroid cells and megacariocytes) were detailed at both morphology and immunophenotypical levels in all groups expect in the frozen series, where the apoptotic degenerative tissue was observed.

In the single cases (1/11, 9%) of samples taken at frozen temperature resulted fully interpretable; cellularity and immunophenotypical characters such as distinction in between myeloid and eritroid components were maintained.

The rest of the frozen series (9 cases out of 11, 91%) allowed visualization of the overall bone marrow cellularity, without distinction of the single components.

In the prospective *day-by-day* decalcified series 7/10 (70%) of cases were interpretable for histopathology. Cellularity and detailed cellular components (mielo-erytroid distinction and megacaryocytes) by using immunohistochemistry were distincted in 8/10 (80%). Immunophenotypical analysis may be well interpreted on tissue after *slow* decalcification and fixation within a 8-14 range days.

Frozen samples are not recommended for histopathological evaluation and subsequent immunophenotypical analysis, due to diffuse absence of vital tissue. H&E staining, due to apoptotic amorphous tissue which do hybridize the proteins with false positive results when immunohistochemical analysis was performed.

CD71 was assessable as dots at frozen samples, without morphological details, and be evaluable as percentages of cells. CD71 in non-forensic and prospective cases was easily countable.

CD3, CD20 and CD34 did not show any positivity in the frozen sternum, whereas in the remaining series showed immunoexpression according to appropriate specific cells (T and B lymphoid and immature cells).

Myeloperoxidase usually does show aspecific background at frozen samples, whereas CD71 may be distincted and be countable as number of cells.

Figures (from 13-21) and Table summarized aforementioned results.

| Table 1. Bone marrow & sternum: sum up of 36 cases studied and interpretable immunoexpression | | | | | |
|---|---|---|---|---|---|
| Case number | | Myloperoxidase | CD71 | CD61 | CD34 |
| | **frozen stored setting** | | | | |
| 1 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 2 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 3 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 4 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 5 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 6 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 7 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 8 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 9 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 10 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| 11 | not evaluable | false positive | absence of expression | absence of expression | false positive |
| | **prospective decalcified & fixed *day-by-day* setting** | | | | |
| 12 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 13 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 14 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 15 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 16 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 17 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 18 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 19 | not evaluable | evaluable | evaluable | evaluable | evaluable |
| 20 | not evaluable | not evaluable | not evaluable | not evaluable | not evaluable |
| 21 | not evaluable | not evaluable | not evaluable | not evaluable | not evaluable |
| | **non-forensic setting** | | | | |
| 22 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 23 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 24 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 25 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 26 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 27 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 28 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 29 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 30 | not evaluable | not evaluable | not evaluable | not evaluable | not evaluable |
| 31 | not evaluable | not evaluable | not evaluable | not evaluable | not evaluable |
| 32 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 33 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 34 | evaluable tissue | evaluable | evaluable | evaluable | evaluable |
| 35 | evaluable tissue | evaluable tissue | evaluable tissue | evaluable tissue | evaluable tissue |
| 36 | evaluable tissue | evaluable tissue | evaluable tissue | evaluable tissue | evaluable tissue |

**Figure from 13 to 22**

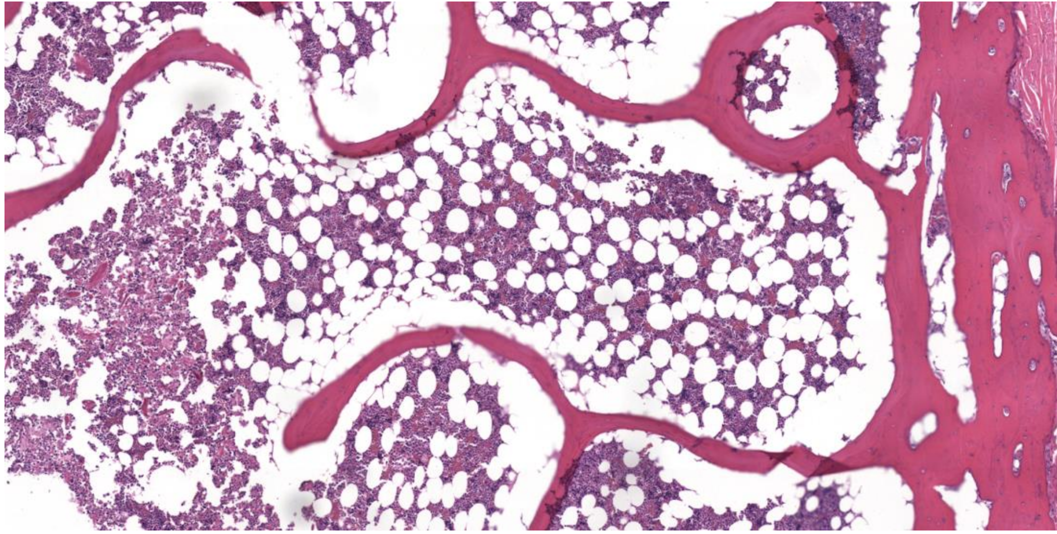

**Figure 13:** H&E bone marrow (decalcification and fixation, 10 days) (day-by-day slow decalcification)

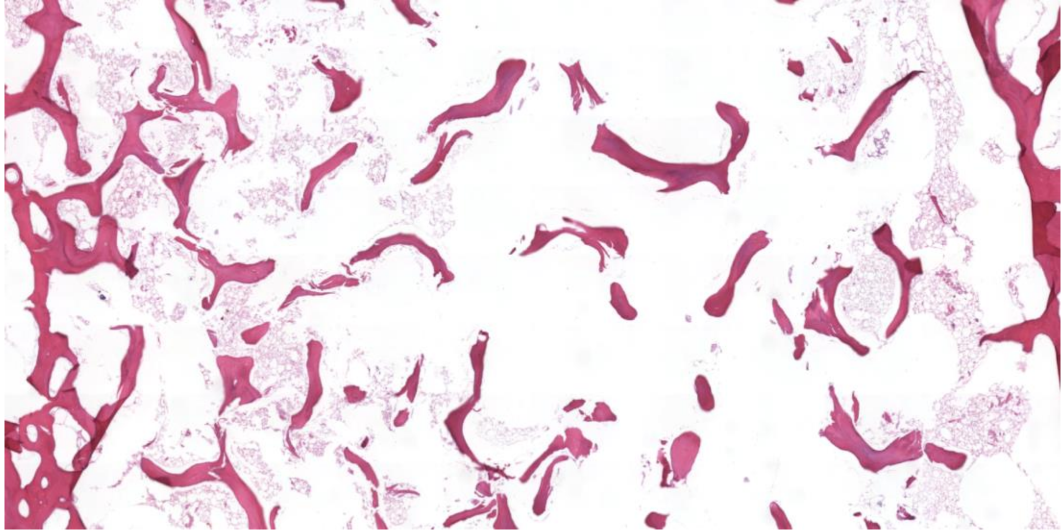

**Figure 14**. H&E bone marrow with day-by-day decalcification without appropriate fixation

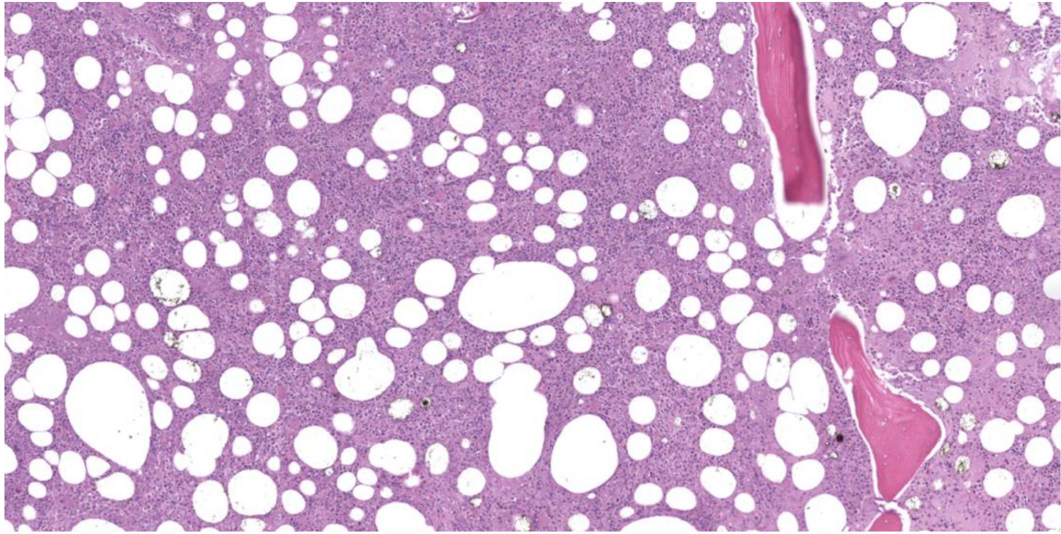

**Figure 15**. H&E staining bone marrow with apoptotic phenomena (day-by-day decalcification and fixation, 4 days)

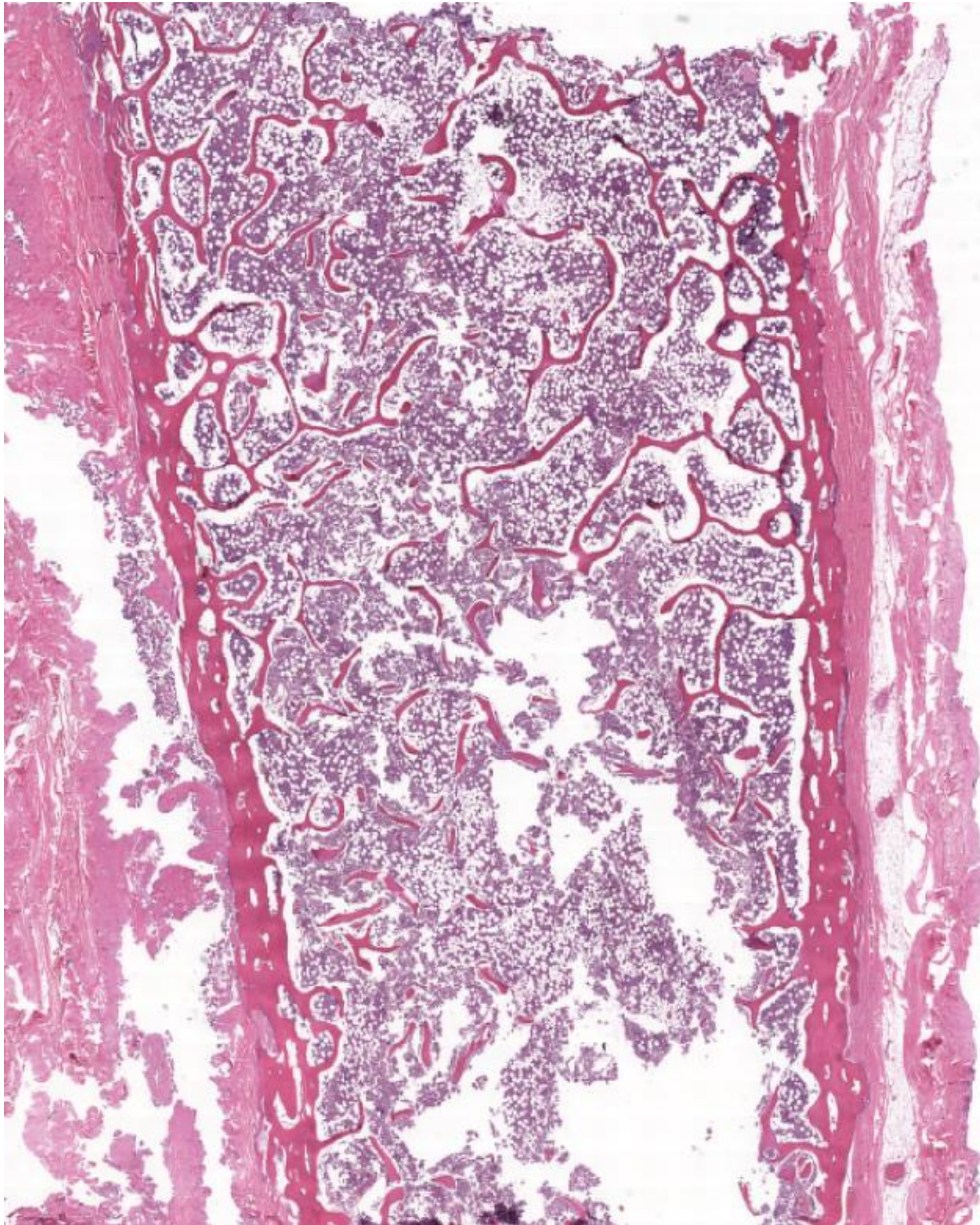

**Figure 16**. H&E bone marrow with day-by-day decalcification process (9 days).

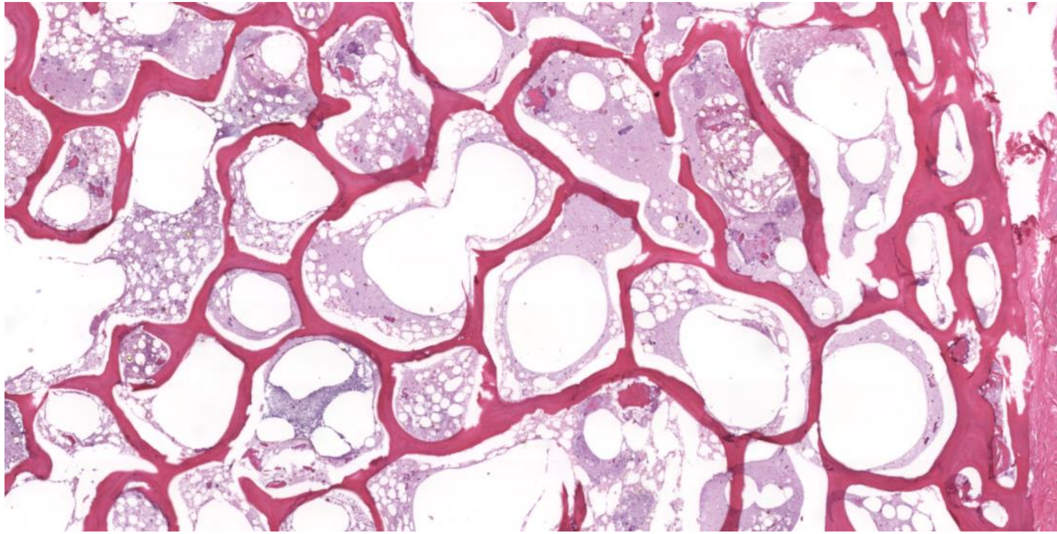

**Figure 17**. H&E with appropriate decalcification but absence of appropriate fixation (6 days)

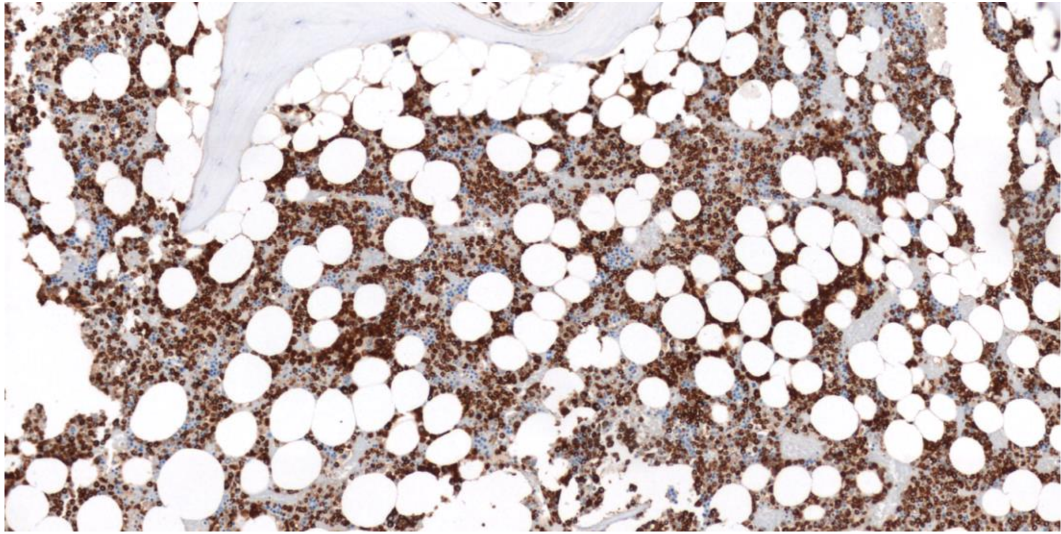

**Figure 18**. Myeloperoxidase (MPX) expression in bone marrow (decalcification and fixation, 14 x) (10 days).

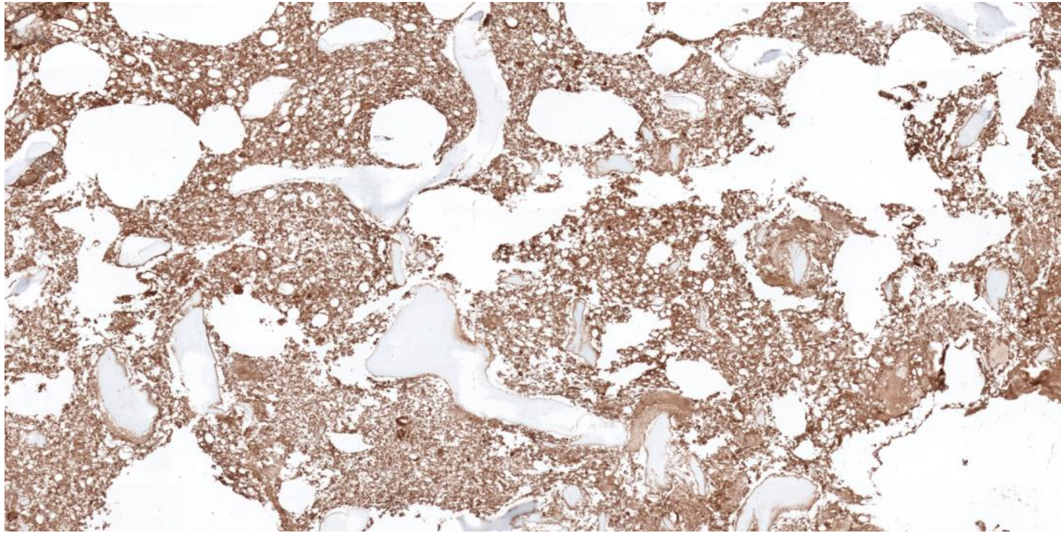

**Figure 19**. Myeloperoxidase (MPX) in bone marrow with background (false evaluation of positive immunoexpression)

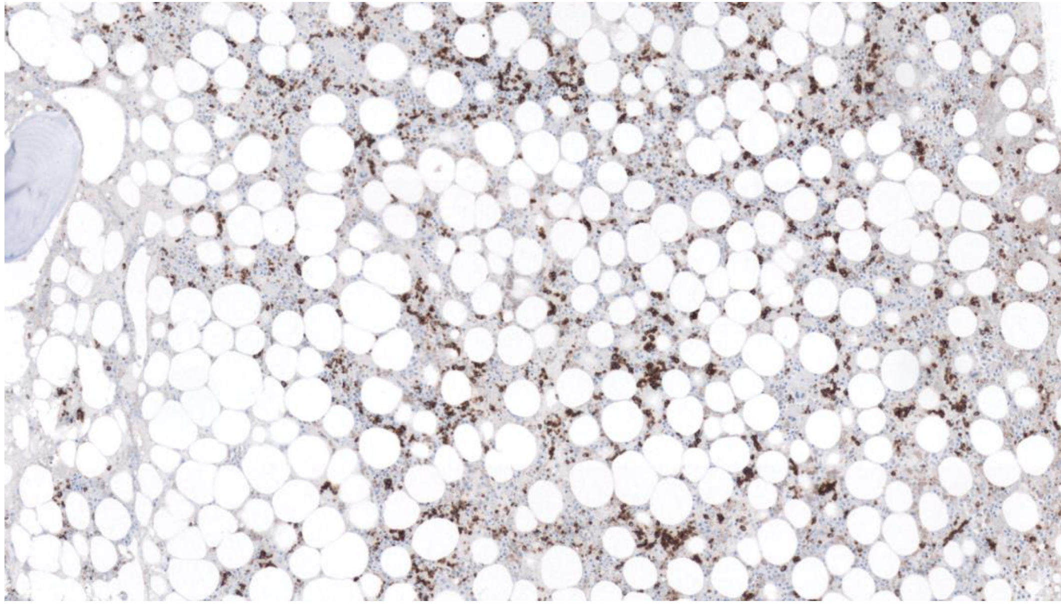

**Figure 20**. CD71 magnification 11x. Cellular specific elements are easily evaluable

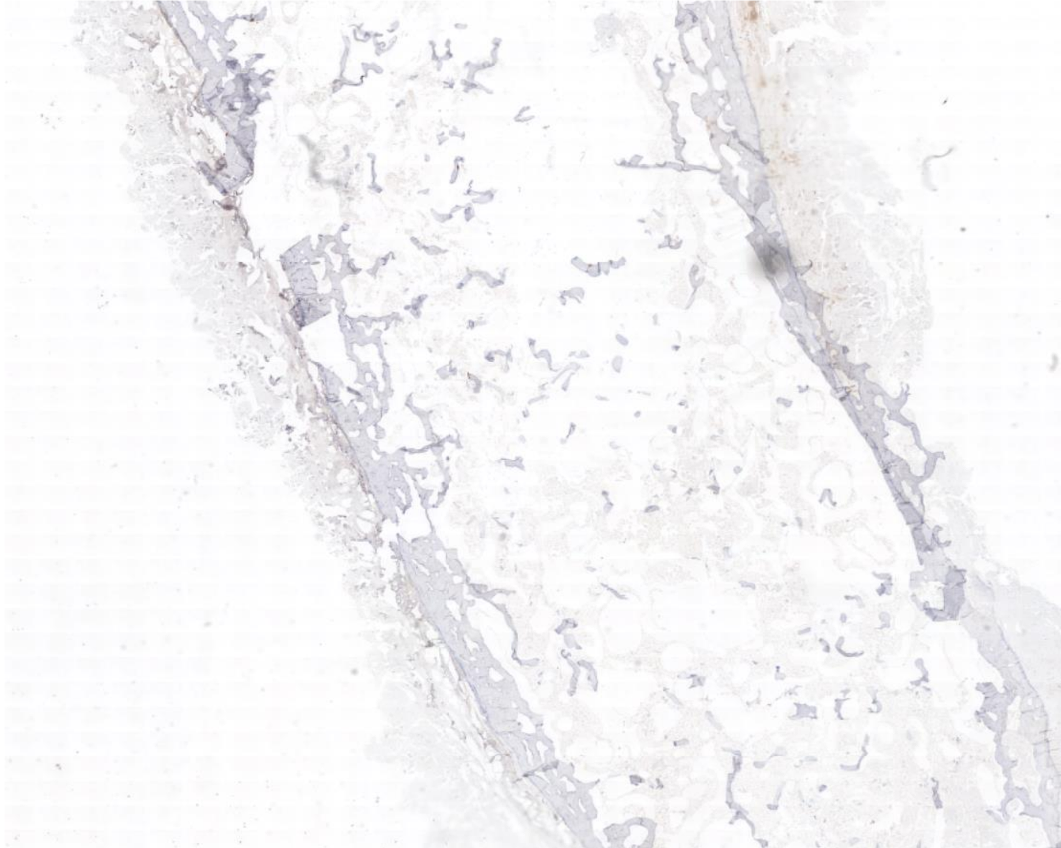

**Figure 21**. Frozen bone marrow: absence of immunoexpression (lack of tissue available)

## 2.4 Discussion

The present study showed that:

1) slow *day-by-day* decalcification process usually let the bone marrow from sternum after forensic autopsies, be morphologically interpretable;

2) immunophenotypical analysis may be performed on tissue after slow decalcification and fixation usually within 8-14 days as appropriate range of sensibility;

3) frozen samples from sternum are not recommended for bone marrow histopathological evaluation.

At routine level, the introduction of fast diagnostic tracks in many areas (i.e of oncology), the traditional processing of bone marrow biopsies (BM), requiring either resin embedding or lengthy fixation and decalcification, is due to an upgrade. Thanks to a growing number of new commercially available tissue processors, microwave-enhanced processing is becoming a standard tool in the pathology laboratory, allowing rapid fixation and decalcification of BM with preserved morphology and antigens. The use of a commercially available EDTA-based decalcification fluid in combination with the LOGOS J, a closed microwave-enhanced tissue processor, for overnight fixation, decalcification, and paraffin impregnation of the BM. This allows next-day reporting without impaired morphology or immunohistochemistry, and even improved DNA quality of the BM. The aim of our study was not to reduce time of reporting, differently was to manage sternum and preservation of bone marrow to obtain sensitive and specific information at morphology.

Systematic investigation of bone marrow samples (BM) in case of forensic autopsies is not yet a routine procedure; most of the studies published in this field have described the use of BM as an alternative tissue in toxicology analyses to analyse changes after the abuse of drugs or alcohol, and in cases when blood samples are unavailable such as skeletonized, bloodless or putrefied cadavers. BM is also used in forensic genetic analysis and in diagnosis of drowning using the diatom test. In the forensic literature there are not many studies that deal with the systematic analysis of the bone marrow to establish a cause of death. [Roll P. at al, 2009; Hellerich U. et al, 1992; De Lima M.A. et al, 2004; Tattoli L. et al, 2014]. Single case reports

are also reported describing a sudden death due to acute and chronic leukemia unknown during life [Umezawa A. et al, 1990; Saka K et al, 2007].

From a review of the literature there are only two studies that focus on the importance of the systemic study of bone marrow as useful tool for obtaining the cause of death.

In the study conducted *by Roll et* al and published in 2009, out of 5740 forensic autopsies in 225 cases (3.92%) a bone marrow sample was taken. The sampling was carried out when an enlarged spleen was found at the sectarian examination, when bleeding disorders were visible, in case of suspected poisoning, in case of suspected septicaemia or when the cause of death was not clear. Bone marrow samples were taken from 12 hours to 140 hours post-mortem from the anterior iliac crests, and were immediately fixed in a 10% formalin solution neutral buffer. In collaboration with the Institute of Pathology, Medical University of Graz, the fixed specimens were further elaborated. These probes were stored in 10% buffered formalin solution for about 24 hours. Subsequently, 5-8 mm cross sections were cut with a special one water-cooled saw and repaired again in buffered formalin for a further 24 hours. Then the tissue samples were incorporated, decalcified, paraffined embedded, cut to 5-6 micron and stained routinely with H & E, Giemsa, silver reticulina stain and blue of Prussia. In selected cases, when there was a suspected neoplasm, immunohistochemical analyzes were performed with antibodies to CD3, CD5, CD20, CD23, light chains (kappa elambda), heavy chains (IgA, IgG, IgM and IgD), myeloperoxidase, CD15, CD33, CD34, CD68. Of 225 investigated BM biopsies they are were able to detect 40 cases with neoplastic disorders not known prior to death. All these BM probes were representative for diagnosis and showed, despite the fact that the BM specimens were extracted up to 140 h post-mortem, surprisingly few autolytic changes when the sections were compared to those of other organs from the same corpse. This good state of BM preservation, due to good conservation of corpses and optimal processing of BM specimens, allowed besides routine and special stains even immunohistochemical investigations with various sensible antibodies.

All cases diagnosed as myelodysplasias showed in addition to dysplasias and atypias within all haematopoietic cell lines marked increase of iron deposits, typical

for these diseases [Frisch B. et al, 1985; Cortelezzi A et al, 2000, Orazi A. et al, 2007]. Within the group of non-neoplastic BM changes the majority showed signs consistent with septicaemia or intoxications [Roll P. et al, 2009] confirming the findings during forensic autopsy. The only histopathologically relevant organ providing a correct diagnosis of postpartum thrombotic thrombocytopenic purpura showing hyaline thrombi in small vessels was the BM [Budde U. et al, 2004]. The authors concluded that it seems necessary to investigate the BM histopathologically if: the forensic pathologist cannot detect "real" cause of death during autopsy, pathological blood count is known or an enlarged spleen is found, septicaemia is established or suspected, fever of unknown origin is reported, suspected or proofed intoxications are documented, and there were symptoms of haemorrhagic diathesis or bleeding tendencies, such as extensive petechias, gastrointestinal or cerebral bleedings or missing or vague livores (signs suspicious for a bleeding disorder).

Another study conducted by *Tattoli et al*, reported that hematopoietic tissue is sensitive to external influences, such as dietary restriction, malnutrition, chronic inflammation, toxicity, and proliferative or neoplastic disorders. Cell differentiation also occurs as a result of several stimuli such as infections or other systemic diseases. In their study *Tattoli et al.* collected a BM samples from cases (n = 73) of medicolegal autopsy performed at the Department of Legal Medicine in Bari (Italy) and at the Institute of Legal Medicine and Forensic Sciences in Berlin (Germany).

BM was sampled from the first part of the sternum, in the mid-portion, by both needle aspiration and biopsy. BM aspirated (2 -5 ml) was collected in a 10 ml syringe connected to a BM needle by piercing the sternum. Approximately 0.25 to 0.5 ml of aspirate were smeared on slides without the use of anticoagulant. Once dried, they were stained with the May-Grunwald-Giemsa and then examined (preferably using an immersion oil, 100 lens in a light microscope). Biopsies from BM trephins were performed on same sternum portion with biopsy needle. In collaboration with the Department of Pathology, samples were fixed in 4% buffered formalin solution for approximately 24 hours. Subsequently, the samples were decalcified, included in paraffin, cut into 5 mm slices and stained with hematoxylin and eosin ( &E). In selected cases immunohistochemical analyzes were performed. The authors found changes in the composition of the MB in relation to the age of

the subject. There was a progressive loss (after 30 years) of hematopoietic tissue and a corresponding increase in the amount of adipose tissue. They showed that the anatomical structure of BM samples showed less autolytic changes than those in other organs. In BM aspirates tissue autolysis was also related to the increasing post-mortem interval (PMI): nuclei gradually shrank and darkened and the cells tended to coalesce; vacuolar degeneration of the cell cytoplasm, which became eosinophilic, the granulocytic cells underwent necrotic modifications of their granules. The authors found changes related to neoplastic diseases and alterations not related to neoplastic diseases; for example in cases where the subjects died of sepsis the BM simples showed a prevalence of neutrophils and their precursors. In fact, there was an increased cellularity with a large number of myeloid precursors, sometimes with a small aggregate of mature neutrophils. Some cases of viral infections showed a reduced neutrophil maturation with a predominance of immature cells, while the modifications mainly correlated with alcohol abuse were hypoplasia, with a lower degree of maturation and, typically, the absence of cells near the trabeculae.

In four cases, BM cell modifications could be indicative of anaphylactic reactions. In 28 cases used as controls, no changes in BM cellular morphology were observed in either the aspirates or the biopsies. The authors concluded that the sternum may offer the ideal matrix from which to obtain morphological evidence of what actually happens in bone marrow. The sternum has the advantage of being more readily accessible during autopsy, as compared to other sites like the iliac crest, femur, ribs, vertebral bodies. These results suggest that BM investigation could provide additional reliable information about the duration of the inflammatory process, reflecting the patient’s ability to respond to external stimuli. The degree of proliferation and differentiation of particular lineages of haematopoietic stem cells is known to be secondary to the production of cytokines which either block or stimulate the BM cells, as a result of infections or other systemic diseases which can be studied by cell morphology analysis. In fact, the recent literature has underlined the role of post-mortem chemical and immunological mediators in determining the manner of death, especially when morphological signs of disease are lacking [Santangelo S. eta al, 2001; Solarino B et al, 2009].

Furthermore, in literature there is considerable evidence that the BM plays a key role in allergic inflammation by inducing an increased production of inflammatory cell progenitors particularly in cases of drug hypersensitivity or allergy [Wood LJ. et al, 2002; Dinger MR et al, 2007].

In this frame, our study showed interesting data that let the BM be play a role in at least a subset of forensic purposes.

The single comprehensive systematic study concerning BM histology from forensic autopsies reported 30 cases with BM changes after chronic alcohol consumption (Roll et al.). In a smaller study, BM findings of 5 necropsied cases of drug addicts revealed titanium pigment within body tissues including the BM. Single case reports on acute and chronic leukaemias undiagnosed during lifetime but detected by postmortem. *Roll et al*. documented and worked up demonstrating the importance of histopathological BM investigations in various conditions. Of 225 investigated BM biopsies they were able to detect 40 cases with neoplastic disorders not known prior to death. All these BM probes were representative for diagnosis and showed, despite the fact that the BM specimens were extracted up to 140 h post-mortem, surprisingly few autolytic changes when the sections were compared to those of other organs from the same corpse. We are in agreement with findings from Roll et al. due to the fact that our data showed a good state of BM preservation, due to good conservation of corpses and optimal processing of BM specimens in the prospective studies. In the manuscript by Roll et al. the post-mortem discovered neoplasia turned out to be of great importance for the responsible clinicians with regard to liabilities for medical malpractices.

Tattoli et al. collected sternum due to the limit of poor samples obtainable by iliac aspiration, especially in post-mortem work-up. In the sternum, BM remains haemopoietically active until late in life, and shows an increase activity more readily than many other bones during regeneration. The sternum may offer the ideal matrix from which to obtain morphological evidence of what actually happens in bone marrow, avoiding the risks both of hypocellularity and of post-mortem involution. Moreover, the sternum has the advantage of being more readily accessible during autopsy, as compared to other sites like the iliac crest, femur, ribs, vertebral bodies. With this

technique, the specimen quality is a remarkable aspect, because a diagnostic interpretation is possible only if morphologically detectable cells are available. In all the specimens evaluated, Tattoli et al reported that the age-related changes of BM were evident, as reported in the pertinent literature. It was possible to analyse all the probes, even if BM samples were taken up to 198 h after death; the BM anatomical structure was still detectable in a cadaver at 32 days after death. As compared with other organs, the integrity of BM despite putrefaction phenomena was evident in histological sections, and the lineage precursors were quite recognizable even at an increasing PMI. In our study we obtained the best immunophenotypical analysis after slow decalcification and fixation usually within 8-14 days in comparison with data from Tattoli et al. Decalcifier I is a unique decalcifying solution that has the ability to fix and decalcify at the same time. Needle biopsies may be completely fixed and decalcified in one hour or less, moreover small bone specimens and calcified arteries may be completely fixed and decalcified in approximately 2-4 hours. Tissues decalcified in this solution cut with ease and routine H&E techniques produce excellent stained sections. The Surgipath Decalcifier I-II combination can save valuable time and more importantly, valuable specimens. Boehm et al. describe bone marrow (BM) biopsies, tartrate-resistant acid phosphatase (TRAP) staining as the gold standard for the characterisation of osteoclasts. TRAP is one of the few enzymes that is histochemically detectable on formalin-fixed paraffin-embedded tissue. The study investigated whether TRAP was also able to visualise BM osteoclasts in autopsy tissue. It was hypothesised that, due to a progressive loss of enzymatic activity in osteoclasts post-mortem, TRAP staining could allow the time of death of a patient to be determined. This study showed that BM osteoclasts stay TRAP-positive for 7 days post-mortem, although with markedly reduced TRAP-SIs compared with biopsies. Since TRAP-SIs were not correlated with the duration of PMI, TRAP staining of BM osteoclasts cannot serve as a tool to determine the time of death of a patient. In our study we did not correlate findings with time of death. The same Authors studied the α-Naphthyl acetate esterase (ANAE), enzymes that are histochemically detectable on formalin-fixed paraffin-embedded tissue. In bone marrow (BM) biopsies, ANAE staining highlights megakaryocytes. Authors investigated autopsy BM to determine whether ANAE staining intensity (SI) was

associated with postmortem intervals (PMI, period between death and autopsy), and thus could allow the time of death of a patient to be deduced. Again, these molecules has not been evacuate in our series. They finally reported that ANAE-SIs in post-mortem BM samples were independent of PMI. Thus, ANAE staining of BM megakaryocytes cannot serve as an indicator for time-since-death of a patient. Wie-techa-Posluszny et al. applied a new instrumental approach to the analysis of human bone marrow for forensic purposes. A new screening method for the detection of more than 30 psychoactive drugs in bone marrow was developed and applied to case samples. They finally presented a method that could potentially find an application in forensic analysis. We can not compare our findings with aforementioned, our series did not involve drug abusers.

## 2.5 Conclusions

We concluded that *day-by-day* (*slow*) decalcification process usually let the bone marrow from sternum after forensic autopsies, be morphologically interpretable. Moreover, immunophenotypical analysis such as myeloperoxidase, CD71, CD61 and CD34 may be performed on tissue after s*low* decalcification and fixation usually within 8-14 days with interpretable assessment. Finally, we do not recommend frozen samples for histopathological evaluation in bone marrow.

The above reported considerations lead to the following final conclusion. The sternum collection in forensic autopsies in order to evaluate the bone marrow is not mandatory in all the forensics cases. However it is recommended in all the cases of suspected haematological diseases, sudden deaths and septic deaths. In these cases the forensic pathologist aims to collect a section of the sternum 2x3 cm, which must be subjected to slow decalcification and fixation within 8-14 days. Following this procedure it is possible to obtain interpretable bone marrow preparations suitable also for the immunophenotypical analysis.

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