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CICLO XXVII

PARALLEL BIOCHEMICAL ANALYSIS OF ACTIN AND OSTEOCALCIN

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SUMMARY

Abstract

Actin is a globular multifunctional protein present in almost all eukaryotic cells, where it plays a major role in the formation of microfilaments, one of the principal cytoskeleton's components, and thin filaments, belonging to the contractile apparatus in the muscular cells. Actin's mass is about 42 kDa and can be present as a free monomer (G-actin, globular) or as a part of a linear monomer (F-actin, filamentous). Actin plays an important role in the embryogenesis, in wound healing, in the invasiveness of tumor cells and in their gene expression.
Osteocalcin is a non-collagenous protein found in bone and dentin, secreted by osteoblasts. It is monomeric protein of 5.8 kDa, highly conserved in all vertebrates. Osteocalcin has an important role on bone mineralization and calcium ion homeostasis. Moreover, recent data suggest a possible role of osteocalcin in male infertility, probably related to the enhancement of testosterone synthesis.
The aim of this thesis was to analyse these two very different proteins, with particular attention to their chemical-physical and functional properties, in order to see if they are related each other in a possible way. The study has been conducted by continuously looking at OMIM, GeneCard and Pubmed, in order to find recent and valuables evidences. Particularly, papers relating to the main post-translational modifications have been also taken into account.
The first point in common which has arisen during such analysis is that both actin and osteocalcin show key modifications during any stage of the growth of *Homo sapiens* starting from the pre-natal phase until senility. Several of these post-translational modifications were related to gender differences. Other findings were related to the fact that both proteins undergo different modifications based on ethnicity, and also linked with nutrition, indeed very relevant to the physical function of both proteins. Subsequently, it was observed that both proteins undergo remarkable changes in at least four different pathological states (vascular diseases, diabetes, renal disease and infertility). Moreover, pH seemed to influence in the same way various post-translational modifications of both proteins. Finally, I have found that several xenobiotics, such as some isoflavones (i.e. genistein) or other compunds (cytochalasin D and latrinculine B) may cause changes in the cytoskeleton or may interact with actin-binding proteins and be involved in the osteocalcin gene expression.

ACTIN

Biochemical properties and physiological role

Actin is a globular multifunctional protein involved in the formation of microfilaments. It is present in almost all eukaryotic cells at the concentration of 100 μM (Moments WF 1952). Actin's mass is about 42 kDa in and cell plays a major role in the formation of two kinds of filaments; microfilaments which are one of the principal cytoskeleton's components and the thin filaments that belong to the contractile apparatus in the muscular cells (Greene LE 1984).
Actin can be present as a free monomer called G-actin (globular) or as a part of a linear monomer composed by microfilaments called F-actin (filamentous), both forms are essential for the most important cellular functions (Greene LE 1984). In fact, actin participates to many important cellular processes, among which muscle contraction, cellular motion, the cell and cytokinesis' division, and it is responsible of vesicles and organelles' motion. Actin forms and works for the maintenance of cell junctions until reaching the cell's real form (Doherty GJ et al.2008).
Many of these processes are mediated by actin and membranes' interaction. On the vertebrates there are three isoforms called alpha, beta, gamma (Vandekerckove J et al. 1978,Taylor A et al.1981 and Gunning P et al. 1983).
The α-actin is in the muscle tissues and it represents an important component of the contractile apparatus (Laing NG et al. 2009),while β-actin and γ-actin are found in the cytoskeleton and they interfere with the internal cellular motion (Laing NG et al. 2009).
The three isoforms are conserved during evolution and present very similar,primary structure in Eukaryotes, in bacteria and in archeobacteria (Ku C et al. 2014). This molecule presents a 20% difference in the amino acid sequence among very different species like algae and humans, suggesting that actin presents an optimized protein structure.
Actin plays an important role in the embryogenesis (Rai R et al. 2008), in wound healing, in the invasiveness of tumor cells and in the gene expression (Shannon GR 1973). Many diseases are connected to the gene change that rules the actin production or its associated proteins. These mutations among humans can be responsible for muscular diseases, changes in the heart's size and function, deafness or they can become target of pathogens (Perrin BJ et al. 2010). Actin presents distinctive characteristics like an enzyme's presence that hydrolyzes slowly ATP, and it is necessary to preserve the integrity of the molecule (Galkin VE et al.2003). Actin interacts with many other proteins, therefore playing different functions, some essential for the cell's life (Dominguez R et al. 2001).Myosin is an example of protein that binds actin (Feuer GY et al. 1954). Another example is the vilina, that plays a role in actin polymerization that dependent on the calcium concentration (Bretscher A et al.1980). Actin, present in the cytoplasm, is one of the most abundant protein in eukaryotes, muscle fibers are composed of about 20% actin while other cells range between 1% and 5% (Yumura S. et al.1998).

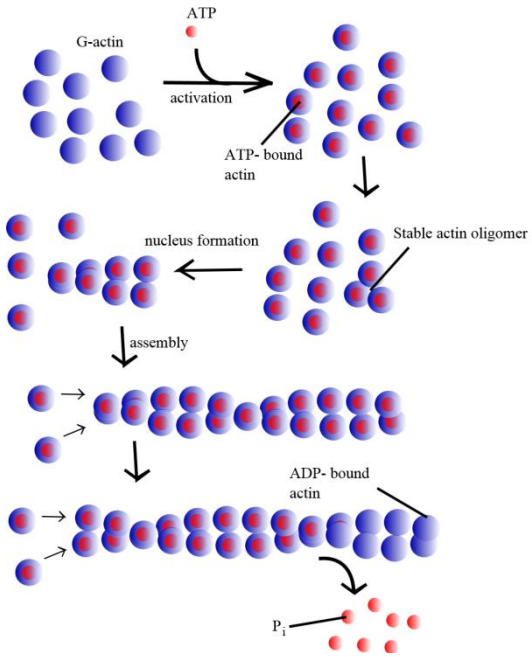
G-actin

G-actin is a globular protein,formed by two domains separated by a cleft, called ATP fold where ATP to Mg^{2+} bind and the enzymatic hydrolysis of ATP in ADP and P takes place.
This fold is a preserved structural motif that can be found also in other proteins interacting with nucleotides triphosphate, as for example the Hsp 70 proteins. G-Actin works only when in this fold there are ADP or ATP, but ATP predominates in cells when actin is present freely (Elzinga M et al.1973 and Collins JH e al.1975).

F-Actin

F-actin has a filamentous structure that can be considered as a single laevorotatory helix with a 166 –degrees rotation around helical axis and with an axial translation of 27.5 A, or a single dextrorotatory helical including in the spacing of 350-380 A.(Holmes KC et al. 1990). Every F-actin filament is composed of polymers with a structure where the subdomains rotate around themselves (Oda T et al. 2009). F-actin polymer seems to have a structural polarity because all the microfilaments subdomains have the same "end",the link for ATP, while the opposed extremity is called "end+".The two filament extremities have at the electronic microscope, a different aspect (Zsigmond SH, 2004). In physiological conditions G-actin monomers change in F-actin polymers; in order to make this possible, the presence of ATP is crucial, as illustrated in figure 1.1.
F-actin helicoidal filament, in the muscle, contains also a tropomyosin molecule. This protein wraps the F-actin helix and during the resting phase covers the active actin sites to avoid actin-myosin interactions and to prevent muscular contraction.

Figure 1.1: *The polymeriztion mechanism for converting G-actin to F-actin. Image taken from "medical gallery".*



Actin folding

Actin can spontaneously acquire most of its tertiary structure, stabilizing to the cytoskeleton and playing an essential role to coordinate the cellular cycle (Martin-Benito J et al. 2002).
The correct folding can take place thanks to of the CCT complex (chaperonin containing TCP-1), defined as the molecular cytosolic chaperone group composed of eight different subunits in a double circle. The CCT acts through ATP-dependent conformational changes that require various catalytic cycles to complete the reaction (Pappenberger G et al. 2006).
To complete the folding, actin has to interact with the prefoldin; PFD3 and PFD 4. These interactions take place in two sites: the first between the residues 60-79, the second between the residues 170-198; the actin is recognized and transported to the CCT. Once reached the CCT, the

actin releases prefoldin (Hansen WJ et al.1999). CCT forms a link with the actin B-apical domain and creates an actin folding sequence (Martin Benito J et al.2007). The first change during the conformational phase place at residues 245-249; during every phase two skeleton protein subunits are linked to the CCT. Specific actin sequences interact with the subunit δ and β-CCT or with ε-CCT. Actin needs CAP protein (catabolite activator protein) to perform the protein folding final stages (Neirynck K et al. 2006).

Actin polymerization

Actin polymerization and depolymerization is necessary for chemotaxis and cytokinesis (Elzinga M et al. 1973). Nucleating factors are necessary to stimulate this protein form's polymerization. One of this nucleation factors is the Arp2/3 complex that stimulates G-actin nucleation (monomeric actin) linking to the filaments. The actin filaments growth is regulated by profilin and thymosin. G-actin is linked to profilin to the exchange ADP and ATP, while G-actin is linked to thymosin to stop the polymerization process (Carlsson L et al.1977 and Goldschmidt-Clermont PJ et al. 1992). The actin filament monomers are aggregated by weak ties, permitting to the structure to ties and release monomers to the extremity according to the environmental stimulation (Elzinga M et Al.1973). ATP is hydrolyzed by a G-actin monomer at the filament level (Elzinga M et al. 1973). According to one hypothesis, hydrolysis occurs randomly and it is influenced by near molecules, other studies, instead, suggest that it occurs through hydrolysis meaning that the process occurs adjacently to other molecules whose ADP has already been hydrolyzed.
In both cases the resultant Pi is not released but it remains for a certain period not covalently tied to actin -ADP creating three actin species in one filament: ATP-actin, ADP+Pi -actin, ADP-actin. Every species quantity depends from filament length and from its condition (Vavylonis D et al. 2005).
Chemical inhibitors of actin:
In nature there are many toxins that interfere with actin dynamics, preventing the polymerization, like cytochalasin D and latrunculine, or stabilizing the molecule, like phalloidin (Cooper JA 1987 and Morton WM et al. 2000). Latrunculine is a sponge's toxin, it ties to the G-actin (Morton WM et al. 2000).
Cytochalasin D is an alkaloid created from mushrooms and it ties to (+) F-actin's filaments' end preventing the addition of new monomers (Cooper JA et al.1987). Phalloidin is a toxin detected in the mushroom tied to the actin monomers interface that is adjacent to the F-actin polymer, preventing depolymerization (Cooper JA 1987).

Primary structure

Actin was observed for the first time in 1887 by WD Halliburton who extracted a protein from from a mixture containg myosin (Halliburton WD 1887). However, the discovery of actin was later attributed to a young biochemical expert named Bruno Ferenc Straub, who showed a new purification technique that permitted to separate a great portion of this pure protein, and Szent-Gyorgyi who described in the same period this new proteic form that later would be named actin (Zallàr A et al. 1989). In 1950, Straub described the ATP presence in actin and its role during the polymerization of actin in microfilaments. This ATP is hydrolyzed in ADP and organic phosphate (Straub FB et al.1989) and it also suggested that this hydrolysis is responsible for muscle contraction (Straub FB et al.1989 and Bàràny M et al. 2001). The amino acid sequence in the actin's

primary structure as represented in graphic 2.1 was determined for the first time in 1973 (Elzinga M et al.1973) .
It contains 374 amino acid residues, the protein's 1 N-terminal is highly acid and it presents a acetyl aspartate in its amino group, while the actin's C-terminal is alkaline and it is composed by phenylalanine preceded by threonine that plays an important functional role (Collins JH et al. 1975). Both the extremes are near the subdomain -1. An anomalous N-methylhistidine is in position 73 (Collins JH et al. 1975).
The concentration of actin in various organisms is quite high, up to 100 µM with a length that can reach 7 nm, while, the isoelectric point changes between 5.4-5.9 (Moments WF 1952).

Figure 2.1 *Actin Primary structure, image comes from Swiss- Prot*

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     10         20         30         40         50         60
MESYDVIANQ PVVIDNGSGV IKAGFAGDQI PKYCFPNYVG RPKHVRVMAG ALEGDIFIGP

         70         80         90        100        110        120
KAEEHRGLLS IRYPMEHGIV KDWNDMERIW QYVYSKDQLQ TFSEEHPVLL TEAPLNPRKN

        130        140        150        160        170        180
RERAAEVFFE TFNVPALFIS MQAVLSLYAT GRTTGVVLDS GDGVTHAVPI YEGFAMPHSI

        190        200        210        220        230        240
MRIDIAGRDV SRFLRLYLRK EGYDFHSSSE FEIVKAIKER ACYLSINPQK DETLETEKAQ

        250        260        270        280        290        300
YYLPDGSTIE IGPSRFRAPE LLFRPDLIGE ESEGIHEVLV FAIQKSDMDL RRTLFSNIVL

        310        320        330        340        350        360
SGGSTLFKGF GDRLLSEVKK LAPKDVKIRI SAPQERLYST WIGGSILASL DTFKKMWVSK
        370
KEYEEDGARS IHRKTF
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Secondary structure
Alpha – binding propeller

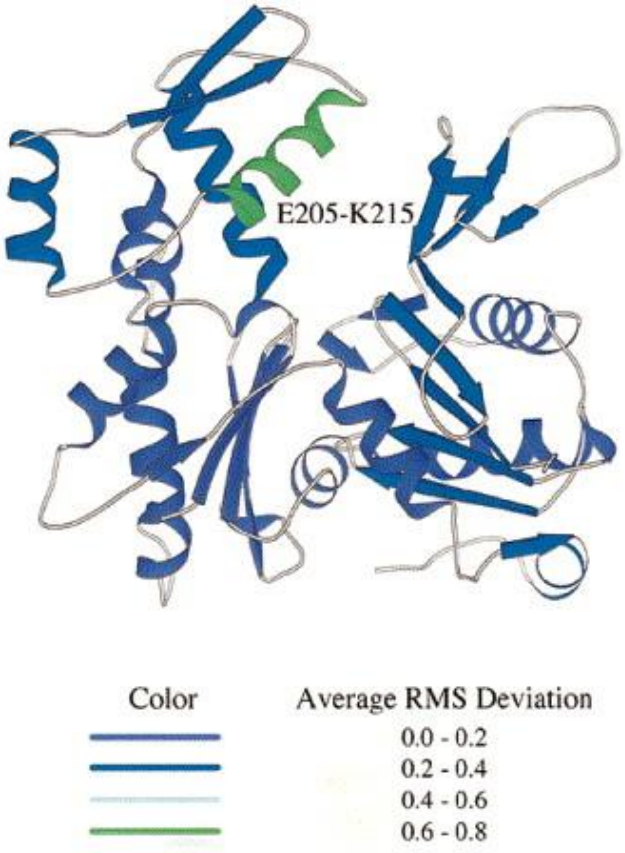


Figure 2.2 *Actin's secondary structure -Domain Motions in actin*
Page R. Lindberg U. and Schutt C.E
Journal of molecular biology
1998, 280:463-474.

Tertiary Structure

The actin's tertiary structure is composed of two domains called the big one and the small one, they are separated by a cleft centered around the link position with ATP-ADP +Pi. Under this slot there is a deep zone called "groove" (Elzinga M et al. 1973).
From topological studies, it results that actin presents the bigger domain on the left side and the smaller on the right side. The small one is divided into two subdomains, so we can find the subdomain in the inferior position where there are the residues 1-32, 70-144, 338-374, and in

superior position the subdomain II that presents residues 33-69 (Kabsch W et al. 1990). The bigger subdomain is divided into two subdomains, the subdomain III that contains in the inferior part (residues 145-180 and 270-337), while the subdomain IV is in the superior part and contains residues 181-269. The exposed areas of subdomains I and III are called ends "barbed" (boned) and the exposed areas to the other two subdomains are called "pointed". The actin's subdomain II is polar (Kabsch W et al. 1990).

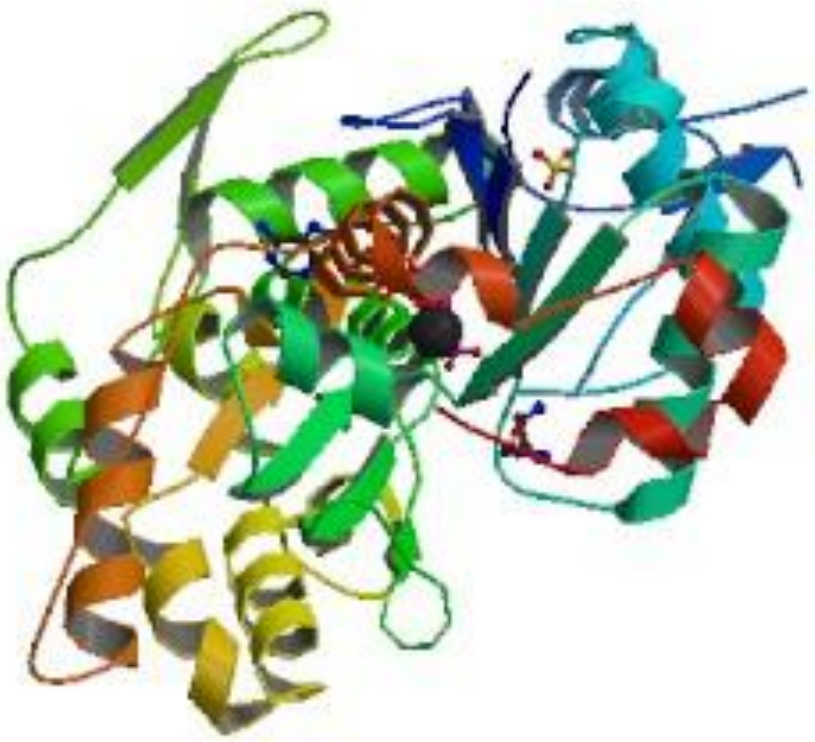
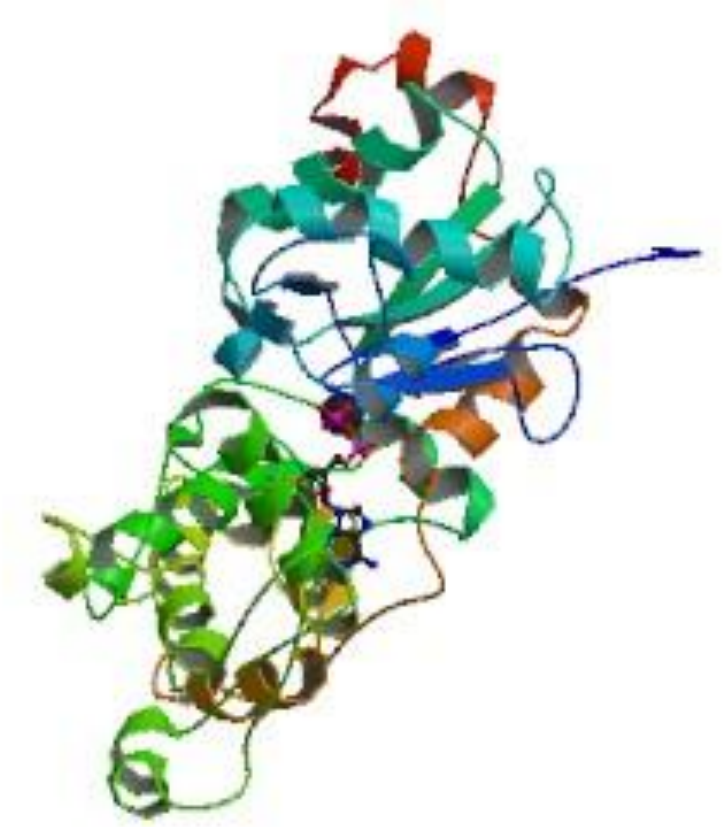


Figure 2.3 Actin's tertiary structure of G-actin observed by Kabsch under X-ray in 1990 *Atomic structure of the actin: DNase I complex*
Kabsch W, Mannherz HG, Suck D, Pai EF, Holmes KC
Nature,1990, 347:37-44.



F-actin

Figure 2.4 *Tertiary structure of F-actin observed by Holmes under X-ray in 1990*
Atomic model of the actin filament.
Holmes KC, Popp D, Gebhard W, Kabsch W
Nature,1990, 347:44-9.

Actin's genes and their expression

Actin in mammals is a unique protein presenting six different 'isoforms' encoded by different genes, as shown in figure 3.1 (Perrin JB et al. 2010 and Engel JN et al. 1981). Every isoform is considerably similar to the others, with only few variations in the amino acid sequence (Oda T et al. 2009).
Moreover, recent studies indicate that different actin's 'isoforms' contribute to the same unique cellular function (Perrin JB et al. 2010, and Engel JN et al. 1981).
Birds and mammals have six genes and each of them encodes a different proteic 'isoform' (Engel JN et al. 1981). Four 'isoforms': α skeletal-actin, α cardiac-actin, α smooth-actin and γ smooth-actin, are expressed in skeleton muscles, cardiac muscles and smooth muscle (Latinkic BV et al. 2002). The others two 'isoforms' β cyto-actin and γ cyto-actin are ubiquitously expressed. All 'isoforms' have very similar amino acids sequences, with similarity of 93%. β cyto-actin and γ cyto-actin, are two 'isoforms' completely conserved in birds and mammals, they differ only for four amino acid residues (Perrin BJ et al. 2010), and contribute in different way to form microfilaments in the cell (Perrin BJ et al. 2010).

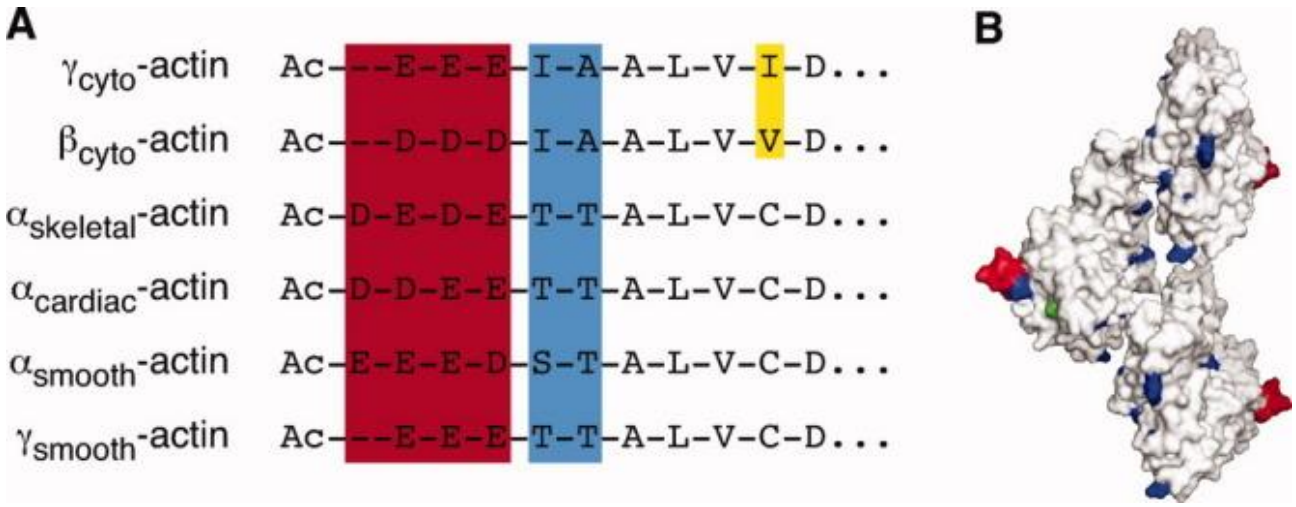


Figure 3.1
a) N-terminal sequences of six actin's isoforms in mammals (Oda et al 2009)
b)3D structure of F-actin showing the red and blue domains reported in figure 3.1.
c)The red residues show variability inside and among the cytoplasmic and muscular isoforms, The blue ones change among cytoplasmic and muscular isoforms, the yellow ones show the change between β cyto-actin and γ cyto-actin the green indicates substitutions among various muscular isoforms.

Actin's 'isoforms', present in mammals, are expressed by different genes that are divided in three classes according to their isoelectric points. Typical actin's gene is composed by about 100 nucleotides 5'UTR, a region of 1200 nucleotides and 200 nucleotides 3'UTR (Hayward LJ et al. 1986). Most of actin's genes are interrupted by introns in 19 different portions. Gene's family is well preserved and it permits a correct analysis about various introns (Hayward LJ et al.1986).
Some prokaryotes have genes like MreB that encode actin's homologous (Figge RM et al. 2004).
ACTA 1 (actin alpha skeletal 1) the gene that encodes α actin is predominant in the human skeleton muscles. It is also traceable in the cardiac muscle and in the thyroid gland and has a DNA sequence

composed by seven exons that produce five relevant transcripts (Gunning P et al.1983 and Taylor A et al. 1988).
These exons may contain point mutations that cause the substitution of amino acid and are responsible for the development of certain pathologies like some myopathies the muscular development associated to weakness, typical facies and breathing difficulties (Nowak KJ et al. 1999).
Generally these mutations are near the ATP binding sites or in the areas involved in the binding with other proteins (Ilkovski B et al.2004).
The enteric smooth muscle's actin is expressed by ACTG2 (actin gamma 2).
The ACTG2 is composed by nine exons, one of which at the 5' extremity is not translated, ACTG2 creates γ actin (Miwa T et al.1991). No transcript mutation of this gene seems to be responsible for pathologies (Miwa T et al.1991).
As for the vascular smooth muscle's actin is expressed by ACTA2 (actin alpha 2) gene. That creates α actin (Ueyama H et al. 1984) and a mutation of MYH11 (myosin heavy chain 11) is responsible of at least 14% of hereditary thoracic aortic aneurysms; moreover, this kind of α actin is known to be a marker for liver cirrhosis (Ueyama H et al.1984).
ACTC1(actin alpha cardiac 1) gene, containg 5 introns (Engel JN et All. 1981) originates α actin, the isoform present in the cardiac mu cvscle (Hamada H et al.1982 and Loon TM et al. 1998).Point mutations of its alleles generate cardiac anomalies' development, like mutation of 1R responsible for cardiomyopathy or hypertrophic cardiomyopathy generated by mutation of 11 type (Matsson H et al. 2008).
ACTB (actin beta) is a complex locus, its sequence contains six exons that can create 21 different alternative splicing transcriptions, called β actin (NG SY et al. 1985).
Consistently with this complexity, its products are well distributed in the organism and are involved in many pathologic processes like infection mechanisms, dystonia youth, nervous system malformation, tumor invasions, carcinomas etc (Gearing M et al. 2002 and Procaccio V et al. 2006).
It was found an additional actin form k-actin that seems to substitute the β actin in the process related to the development of cancer (Chang KW et al. 2006).

Absence or mutations of actin's isoforms

Studies carried out on model organisms, among which Drosophila, C. Elegans and various murine models, have demonstrated that various muscular 'isoforms' play the same cellular functions but have also distinct roles (Wagner CR et al 2002 and Perrin BJ et al. 2010). For example, some experiments demonstrate that mice without α cardiac-actin, die during the embryonic or perinatal development with an important disorganization of cardiac myofibrils (Kumar A et al.1997). Other experiments show that mice knockout for α skeletal-actin appear normal at the birth, but they present weak muscles and they die after nine days (Crawford K et al. 2002). Mice knockout for α smooth-actin, born under a mendelian relationship seem to be vital but they develop contractile vascular defects and anomalies in regulation of blood pressure (Crawford K et al. 2002). Knockout γ smooth-actin has not been reported yet.
The over-expression of some actin isoforms can be compensatory of other knockout forms, but because every isoform performs similar but distinct roles, the mice, in many cases, present anomalies and brief life as shown in figure 3.2 (Perrin BJ et al. 2010).
α cardiac-actin and α skeletal-actin are 99% identical and they present the same expression pattern. These similarities suggest that a α skeletal-actin's deficit can be solved by a transgenic therapy,inducing the over-expression of α cardiac-actin (Vanderkerchove J et al.1986 and Nowak KJ et al. 2009 and Jaeger MA et al.2009).
Cytoplasmic 'isoforms' β cyto-actin and γ cyto-actin, as already reported above, are almost identical proteins that differ for only four amino acids, all located in the N-terminal zone (Perrin BJ et al. 2010).

Knockout mice for β cyto-actin die early during the development for a not characterized defect (Shawlot W et al.1998 and Smerling D et al. 2005), instead knockout mice of γ cyto-actin are vital and can live until adult age except for some subjects that at birth; are smaller than the murine wild-type and can die in adult age (Belyantseva IA et al. 2009 and Bunnell TM et al. 2010).
The potential loss of γ cyto-actin in life causes similar problems to cells. For example the loss of these two cytoplasmic isoforms in the sensory hair cells of the ear, called stereocilia, lead to deafness in advanced age (Belyantseva IA et al. 2009 and Bunnel TM et al. 2010).
Moeover, these two forms are important for the formation of the sarcomere in the muscle (Sonneman KJ et al. 2006).
In all the murine models studied so far, from the absence of one isoform it is clear that the total concentration of actin is the same, probably for the over expressions of other similar "isoform". Sometimes the phenotypes observed can be attributed to change in the actin composition (Perrin BJ et al.2010).
As for mechanisms and various "isoforms"' functions, two hypothesis are supported to explain how the different actin forms can perform different cellular functions (Tzima E et al. 2000 and Wagner CR et al. 2002).
In the first hypothesis the actin-binding protein could tie to determined "isoform", in fact it is observed that profilin (Larsson H et al.1988), cofilin (De La Cruz EM 2005) thymosin b4 (Weber A et al. 1992), I-plastin (Namba Y et al.1992), ezrin (Yao X et al. 1996) and β CAP73 (Shuster CB et al. 1996) could distinguish among the isoforms of muscular and cytoplasmic-actin.
Annexin 5a prefers to tie γ cyto-actin and β cyto-actin (Perrin BJ et al. 2010). The second hypothesis can be attributed to the fact that different isoforms of actin are located in different subcellular zones, maybe for a transcription mechanism or for a mechanism lead by interactions with actin-binding protein; in fact, the cellular localization of different isoforms actin is observed in various types of cells (Perrin BJ et al. 2010).
The skeleton muscle seems to supply a clear example of differential localization of actin "isoforms"; in fact, α skeletal-actin is limited to the thin filaments sarcomere, while γ cyto-actin is not found in the thin filaments of muscular cells but it's observed in other muscular cellular structures as, for example, in filamentous structures adjacent to the sarcolemma and it is the only isoform detected in muscular zones that is found between sarcolemma and the z-disk zone and in a zone adjacent to the z-disk (Craig SW et al.1983 and Pardo JV et al. 1983).
More over γ cyto-actin is distributed in all the structures that contain actin while the β cyto-actin is more polarized; this difference can be attributed to a nucleotide founded in the 3'UTR sequence of the transcripts β cyto-actin and not in γ cyto-actin (Condeelis J et al.2005).
This last difference is essential for the β cyto-actin has functional consequences in different cellular types; in fact this isoform is essential for a correct development at a neuronal level, it affects the fibroblasts' development, and myoblasts (Yao X et al. 2006).
New biochemical studies indicate that actin properties can change according to the mix of isoforms in the filament (Bergeron SE et al.2010). Recently it was observed that β cyto-actin polymerizes and depolymerizes faster than γ cyto-actin isoform (Bergeron SE et al. 2010). Different actin mix, can have different biophysics properties or distinct interactions with the regulators stability like cofilin (Perrin BJ et al.2010). We can conclude that modifying the relation between different isoforms of actin inside of a filament can tune its properties to satisfy the specific requirements of different cells subcellular structures (Perrin BJ et al.2010).

Modification or absence of various actin isoforms

Figure 3.2 *Genes and phenotypes related to the six actin "isoforms"*

isoform	Gene	phenotype
α skeletal-actin chromosome 1	Acta1	Puppies die at 9 days, muscular weakness
α cardiac-actin chromosome 15	Actc1	Puppies die like embryos or just born disorganization myofibrillar
α smooth-actin chromosome 10	Acta2	Various defects about contractility and blood pression
β cyto-actin chromosome 7	Actb	Lethal at embryonic level
γ cyto-actin chromosome 17	Actg1	Reduced vitality, small dimensions, progressive deafness
γ smooth-actin chromosomee 2	Actg2	Partial risk of lethality, cardiac defects

Post-translational modifications

Actin, after its maturation is subject to many post-translational modifications (Terman JR et al. 2013).
Among the best known we can list (Figure 4.1)
-Acetylation of the actin N-terminal (Cook RK et al.1991). This modification is necessary to facilitate actomyosin interactions in muscles and to determine actin ubiquitination (Cook RK et al. 1991). This modification is not essential in lower eukaryotes (Cook RK et al.1991). Moreover it seems that acetylation processes are mediated by deacetylase and deacetyltransferase, as shown by the fact that HDAC6 histone deacetylase associates itself to the actin and participates to the rearrangement in vivo of the protein (Obrdlik A et al. 2008).
Acetylation is fundamental to modulate the role of actin in movement's cells (Vandekerchove J et al. 1978).
-ADP-ribosylation pathogenic bacteria express various ribosyl-transferase that transfer portions of ADP-NAD to specific residues of actin (Sheterline P. et al. 1995 and Aktories K et al. 2011). A well characterized ADP-ribosyl-transferase is the one expressed by Clostridium Botulinum C2 toxin. ADP-ribosyl is added in the non muscular G-actin on arginine 177 causing actin destruction, which indicates that this modification inhibit polymerization (Okamoto H et al. 1997).
-Arginylation is a modification decided by arginyltransferase (ATE1), a biologic global regulator (Karakozova M et al. 2006 and Kashina A 2006). This modification in some cases is essential, because if actin is not correctly arginylated the subject will have contractile defects, cardiac-vascular problems, and numerous morphological defects of the neuronal crest (Rai R et al. 2008). In the absence of arginylation, F-actin decreases (Rai R et All. 2008). The protein undergoes internal arginynilation and later it undergoes hydrolysis at the N-terminal level (Karakozova M et al.2006 and Rai R et al.2008 and Kurosawa S 2012).
-Cross-linking, is likely a modification used by bacteria to modify actin; for example the Vibrio Cholerae causes cells rounding and F-actin loss through actin cross-linking due to enzymes like MARTX (multifunctional autoprocessing repeats in toxin) and VRG G-1 (valine -glicine-repeat G1) it (Russo L et al. 2013).
Actin is covalently modified by sugars. Actin is easily susceptible to O-GlcNAcylation (O-GlcNac). This modification occurs in the cytoplasm and in the nucleus and it is controlled by two well-maintained enzymes that add or remove O-GlcNac according to the received stimuli (Fofana B et al. 2010). This modification regards cardiac and skeletal muscle modulation as well as contraction and protection (Jones SP et al. 2008). Moreover, it is known that glucose that binds to a Lys fragment causes a change in tissue in diabetic patients where sugar molecule causes decrease and it decreases F-actin deforming cells until bringing a locking micro capillary followed by diabetic retinopathy (Solution KN et al. 2001).
Actin methylation on His 73 which regulates the molecule flexibility and stability.
-Methylation in actin occurs also in Lys 326.
-Redox modifications, referred to oxidation and nitrosylation are modifications that occur in actin (Feuer G et al. 1948). In fact it is noted that by adding an oxidant agent globular actin's polymerization can be prevented and at the same time polymerized actin is destroyed (Feuer G et al. 1948). It is concluded that actin is susceptible to redox post-translational modifications inclusive of oxidations, nitrosylation, carbonylation, nitrations and glutathionylation (Feuer G et al. 1948). Clearly this susceptibility to the redox intermediates changes according to the status of the actin especially if it is on filamentous or globular form (Feuer G et al.1948). Also, actin presents 5 Cys that are highly sensible to oxygen, nitrogen and lipid reactive species. For example Cys 374 due to air exposition, aging and icing it causes formation of disulphide bonds among protein monomers

(Feuer G et al.1948). A Cys 272-285 sensible to redox modifications, chich causes decreasing polymerization and an altered interaction between actin and regulator proteins. Also some of the 16 Met residues present in actin are susceptible to oxidations causing functional alterations to actin. (Milzani A. et al. 2000 and DalleDonne I et al. 2002).

His and Tyr residues in actin are,susceptible, to modifications due to redox reactions (Hung RJ et al. 2011). It seems that actin scavengers reactive species to protect the cell against oxidative stress. (Takamoto K et al. 2007). The multi-domain-flavoprotein monooxygenase modifies actin by oxidizing the Met44- Met47 residues, causing F-actin destruction and decreasing polymerization (Takamoto K et al. 2007).

-Actin phosphorylation is one of most well known actin modifications; in fact, there are 35 phosphorylation sites on actin; the best known example is the Tyr53 residue of actin, which interferes with the molecule polymerization (Bender N et al. 1976). Nevertheless, in superior organisms, phosphorylation of actin is not yet understood.

- Actin is susceptible to sulfation even if it is not clear how this occurs (Schick BP et al. 1994). Sulfotransferases can use and bind F-actin to mobilize and permit sufation of hydroxysteroid like cholesterol in specific intracellular destinations. Numerous covalent modifications such as succinylation and malonylation can be fundamental in regulation in vivo of actin (Walsh CT 2006).

-Actin is ubiquitilated by specific ubiquitin ligases, like MURF-1 (muscle RING-finger protein-1), UBCH5(ubiquitin-conjugating enzyme E2D), TRIM32(tripartite motif containing 32), that decrease the actin levels during the atrophy phase and muscular remodeling, even if myofibrillar proteins, as myosin, seem to protect actin from degradation (Solomon V et al. 1996 and Kudryashova E et al. 2005). Actin wrongly arginylated is also ubiquitilated or may have wrong post-translational modifications. Poly-ubiquitylation and mono-ubiquitylation seem to confer stability and differential subcellular localization of actin. SUMO (small ubiquitin-like modifier) modifications have been identified but their effects are unknown (Kudryashova E et al. 2005).

Actin is also a substrate of enzymes like L-isospartyl methyltransferasi (PIMT) that converts isoaspartyl residues to aspartyl residues.

Actin G and F filaments assembly in vitro seems faster in acid pH.

- PH modifications regulate also some adhesion sites of actin (Schonichen A et al. 2013).

PH effect on focal remodeling of adhesion is mediated in part by talin bound to F-actin (Schonichen A et al. 2013).

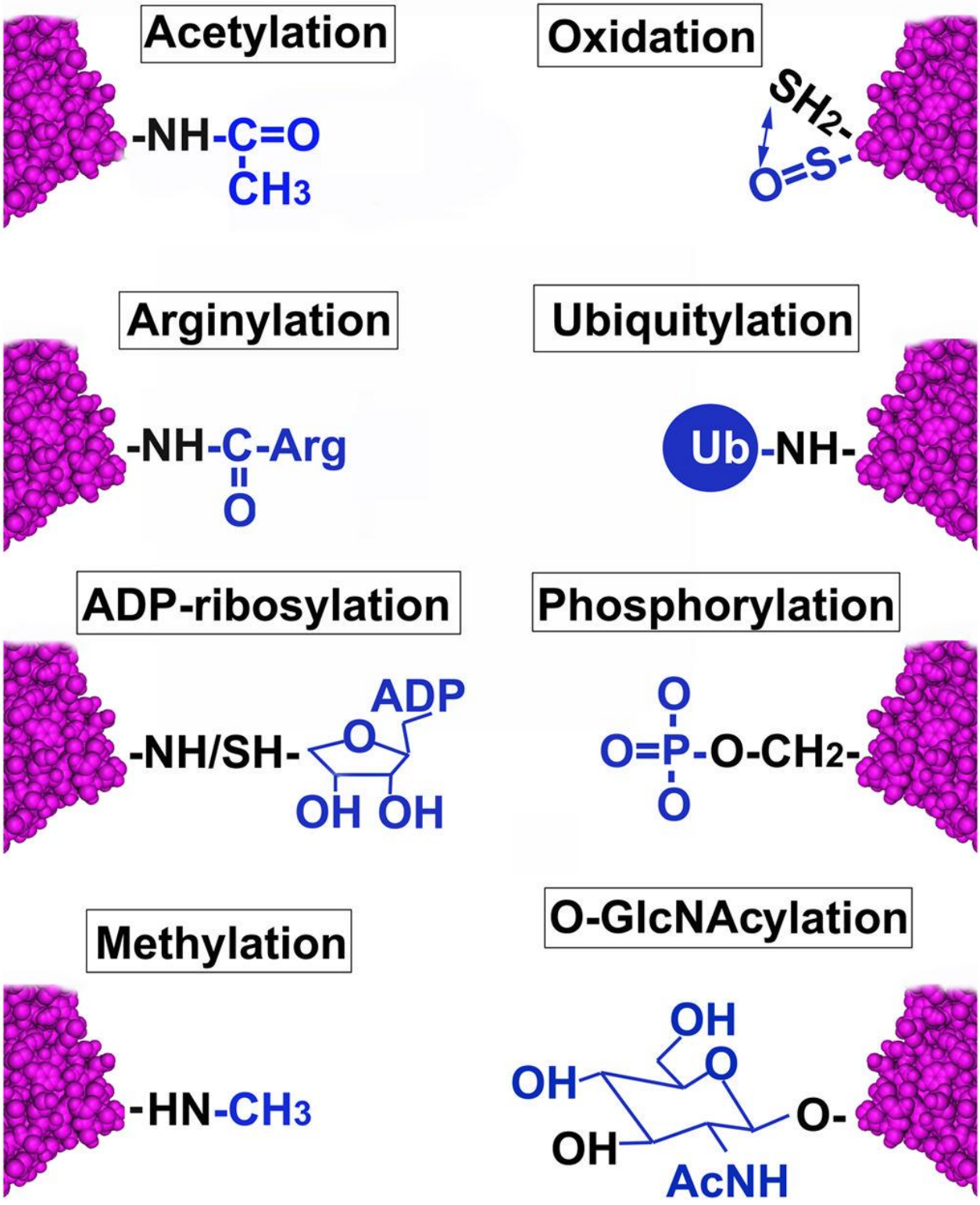
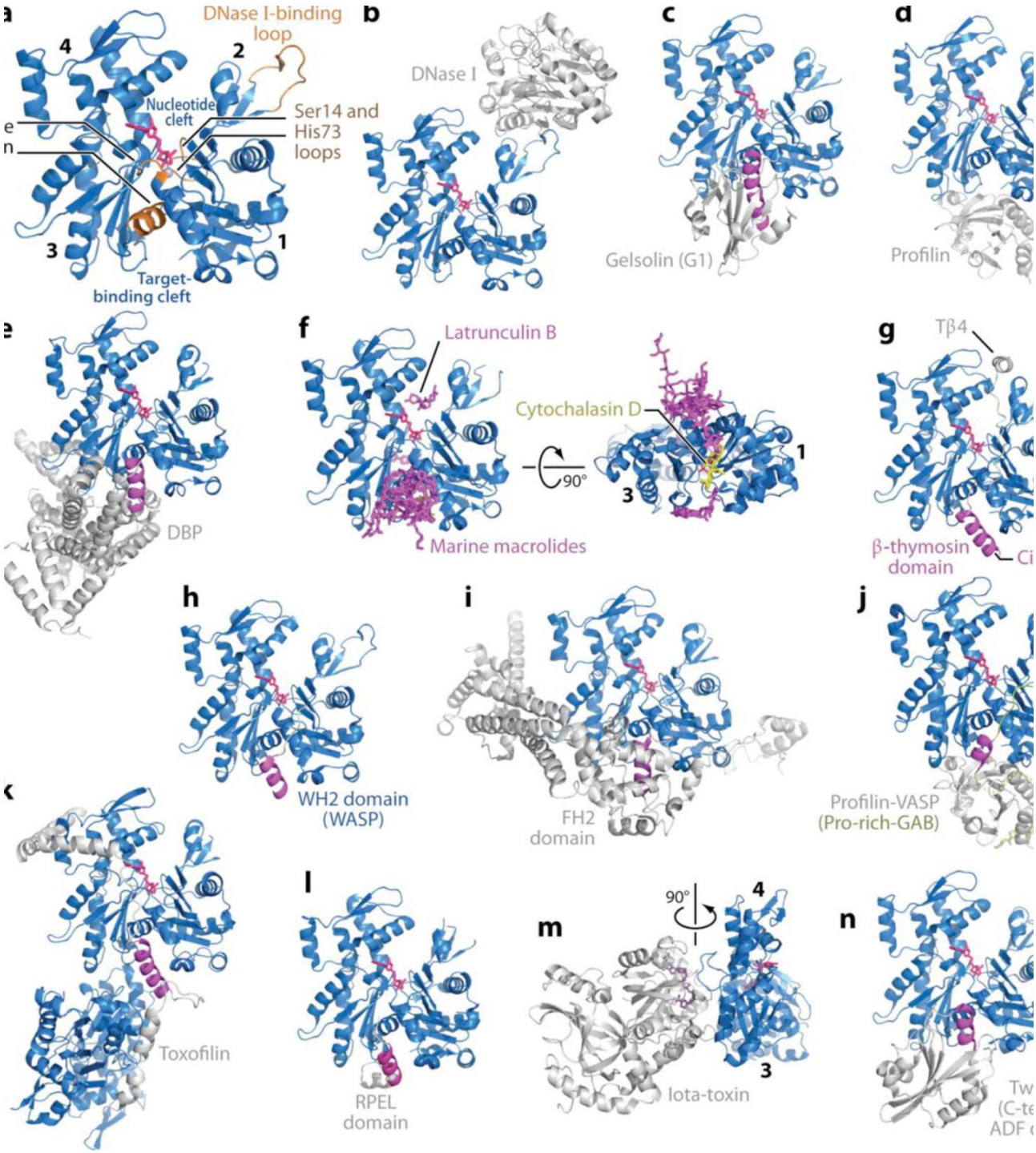


Figure 4.1 *graph illustrating the most important post-translationalmodifications of actin- -The image has been taken from "Chemistries of the major actin modifications".*
Post-translational modification of actin
Terman JR and Kashina A
Curr Opin Cell Biol
2013,25(1) 30-38

Post-translational modifications of actin-binding proteins

It seems right to affirm that actin interacts with other molecules more than every other protein or molecule present in the organism, and this induces post-translational consequences to the protein. A 2011 study reports actin interaction with numerous proteins actin-binding (Dominguez R et al. 2011).
Vertebrates express three kinds of "isoforms" of actin (Engel JN et al 1981
Among famous actin-binding we can find:
-DNase I binds with actin forming a highly affinity complex, even if it is not clear what the physiological role is (Kabsch W et al.1990).
-Gelsolin, that belongs to a actin-severing and actin-capping family, which includes adseverin, vilina, CAPG(capping protein actin filament) and advilin (Silica P et al. 2004).
Gelsolin consists of 6 domains (G1-G6) and it's regulated by Ca2+, phosphoinositoles and a tyrosine phosphorylation. Gelsolin without Ca2+ is in the inactive status seems and it adopts a compact conformation, stabilized from intramolecular interactions in which binding sites for actin are unavailable. The most important domains in gelsolin are G1-G4 that result to be actin-binding (Burtnick LD et al. 1997). In fact the release of Ca2+ produces a conformational change that exposes binding sites of actin to G1-G4 (Burtnick LD et al. 1997). Gelsolin is principally located in the cytoplasm, but one of its longer isoforms circulates in the blood and belongs to a homeostatic mechanism, defined actin-scavenger. In fact, it is noted that after cellular death or cell lesions,actin can be released in the blood flow (Lee WM et al.1992). The actin-scavenger is responsible for depolymerization and removal of actin from circulation(Lee WM et al.992).
-Profilin contributes with the Tβ 4 (thymosin beta-4) which plays a role in regulation of actin polymerization. Thymosin beta-4 is a protein that in humans is encoded by the *TMSB4X* gene (Gomez-Marquez J et al. 1989). The protein thymosin beta-4 , is important because after a heart attack, might reactivate cardiac progenitor cells to repair damaged heart tissue (Smart N et al. 2011).
-ADF-cofilin is particularly present in the eukaryotes organisms is normally involved in the recycling of actin monomers that come from depolymerized filaments during cytoskeleton turnover (Bernstein BV et al. 2010). This family comprises ADF (actin depolymerization factor) cofilin-1 in non muscular cells, cofilin 2 in the muscular cells and twinfilin that contains two ADF domains (Paavilainen VO et al. 2008).
-Macrolide was recently isolated in some marine organism. These molecules interact with actin preventing polymerization (Yeung KS et al. 2002).
Among these macrolide we can find cytochalasin D, a fungal toxin widely used in cellular biology. This one inhibits both association and dissociation of monomers of actin (Yamaha EG et al. 2000). Latrunculine A e B inhibits polymerization and tie to a actin flection (Yamaha EG et al. 2000).
Some human pathogen agents cause problems to the cytoskeleton during infection. Toxofiline binds to G-actin secreted from host cells during invasion, while other pathogen bacteria produce toxins that transfer the ADP-ribosyl group on actin. This is the botulin toxin C2 and the iota-toxin that modify Arg 177 (Goode BL et al.2007, Barth H et al. BG 2008 and Lang AE et al. 2010).
-FH2 modify assembly of actin, it promotes morphogenesis, cellular locomotion and cytokinesis (Galkin VE et al. 2002).
-FH1(formin homology 1) promotes fragments assembly of branched actin and its rich in profilin (Kovar DR et al.2006)

Figure 4.2: Structures of actin and actin complexes
Dominguez R and Holmes KC
Actin structure and function
Annu. Rev. Biophys.
2011,40:169-186.



Catabolism

Actin, after cancer cachexia processes, muscular or cellular atrophy, inflammations, senility, and some pathologies, goes through a degradation phenomenon (Song G et al.1997).
In a 1997 study (Song G et al.1997) it was observed that in PC12 rat cells, in apoptotic phase, actin is degraded (Song G et al. 1997). In fact, in this cell model, normally, actin inhibits DNase I. But when there is intervention of a responsible for apoptotic process the endonuclease seems free to enter in the nucleus and fragments DNA. The actin fragmentation leads to the cytoskeleton break up and the morphological changes occurring during apoptotic process.
Actin site is cleaved at residue Asp 244, which gives rise to a 30 kDa product (Song G et al.1997).
Another 2004 study (Kong Jl and Rabkin SV 2004), instead, indicates that actin polymerization phenomena and polymerization inhibition occur in conjunction with the apoptotic process, leading to a complete disorganization of F-actin (Gourmet CW et al. 2005).
In fact, cytoskeleton disorganization causes cytochrome C release and activation of caspase-9. This causes the caspases cascade activation actin does not inhibit DNase I, that enters in the nucleus and degrades DNA (Enari M et al. 1998).
Three actin-binding,HS1(hematopoietic lineage cell-specific protein-1), cortactine and HIP-55(HPK1-interacting protein of 55 kDa) are substrates for caspase-3 and suffer cleavage during apoptosis (Thomas SG et al. 2007).
In another study it has been proved that the cytoskeleton degradation is induced by lovastatin and it's mediated by caspase-3, caspase-2 (Kong JI et al. 2004).
In 2011, a study about catabolism of actin illustrated how this molecule is the most degraded protein following poly-ubiquitination (Pole C et al.2011).

Variations in *Homo sapiens*: prenatal stage

According to some researches, the post-translational modifications of actin start in intrauterine phase. This provides the correct functions of various organisms and tissues.
A 2008 study (Rai R et al. 2008) indicates that post-translational modifications such as arginylation mediated by arginyltransferase (ATE1) (Balzi E et al. 1990) of actin are essential for the cardiovascular development and for angiogenesis in mammals, especially for what concerns the myocardic structure during cardiac development (Karakozova M et al. 2006 and Kashina AS 2006). This analysis, performed through the characterization of embryonal actin by gel fractionation and mass spectrometry, evidences that α cardiac actin is found in an arginylated form during development. The four arginylation sites works cooperatively in the actin monomer to modulate polymerization and co-assembly of myofibrils (Saha S et al. 2010)
To see how actin is arginylated during in vivo embryogenesis, the different forms of actin have been separated thank to the different of isoelectric point permitting its identification (Rai R et al. 2008).
As for the two arginylation sites isoleucine 87 and the glycine 152. Both are far from N-terminal and for this reason a proteolytic cleavage is probably required before arginylation in order to avoid destroying the molecule'integrity. Mapping of actin arginylation sites permits to verify that all are accessible until the molecule folds, but that they are not accessible after polymerization, and so probably the modification occurs after folding but before polymerizing (Rai R et al. 2008). These modifications facilitate polymerization and myofibrils interaction with others proteins. Thin walls, poor continuity between myocytes and myofibrils and evident pallor in embryo heart of knockout ATE1 mouse. This shows the importance of this post-translational modification in fetus. The two forms of α and β actin are arginylated in vivo (Karakozova M et al. 2006 and Rai R. et al. 2008 and Kusosoka S et al.2012).
Cardiac actin is aginilated to the 50% suggesting that this protein is fundamental for myofibrils integrity in fetus. In this study it was also demonstrated that if the 1 α cardiac actin is substituted

with a muscular enteric “isoform” that can’t be arginylated serious contractile defects during myofibrils assembly and heart development are observed (Rai R et al. 2008).
Lack of arginylation, in fetal mice with a substituted actin evidences also problems in pre-myofibrils where actin polymerizes slowly, and so it impose an anomalous aggregation and it causes a lack of interaction with other proteins for nucleus myofibrils formation to a cardiac level (Rai R et al. 2008).
Post-translational modifications of actin seem to have a fundamental role in neonatal level too. A 2003 study (Shiraishi Y et al.2003) indicates that cupidine (Shiraishi Y et al.2003), a famous actin-binding protein, in baby binds F-actin acting like a particular adaptor. It was observed that cupidin is widely localized in granule cells at the post-synaptic level , in post-natal phase and it decreases in adult phase. In fact, in the cultured granule cells,cupidin is present in younger cultures 7DIV but not in older ones 21DIV.
Another 2012 study (Fediuk J et al. 2012) indicates the various changes of actin at the pulmonary artery level allows the baby to adapt completely to a extrauterine life.
Fetal adaptation to extrauterine life consists in a rapid increase of artery pression, of myocytes length and lumen diameter increase of pulmonary artery. Hypoxia or persistent pulmonary hypertension of newborn (PPHN) prevent the transient postnatal reduction of actin. The functional role of actin in pulmonary artery hypertension was never characterized,even if it seems that actin polymerization is important for myofilaments stabilization and for increasing generation of force. (Haworth SG 1995).
Although this field is fairly unknown, it is observed that both hypoxia and vasoconstriction can contribute to the lack of remodeling of pulmonary artery, in the neonatal hypoxic pulmonary hypertension by interfering with the cytoskeleton reorganization. (Hall SM et al.2000 and Cipolla MJ et al. 2002).
In a study by Fediuk J et al.2012 hypoxia and vasoconstriction are examined as distinct stimuli for actin polymerization, by confronting systemic answers of myocytes in pulmonary artery, of the baby. The aim is to determine different paths that regulate actin polymerization in the pulmonary circulation systemic and to correlate actin polymerization with the force generation induced by thromboxane in arteries with the pulmonary hypoxic neonatal hypertension (Sakurada S. et al. 2003).
In this context it is evident that, although hypoxia promotes actin polymerization through Rho-linked to an arterial level,in this case polymerization of actin is thromboxane-induced in myocytes in pulmonary and artery is independent from Rho (Sakurada S. et al.2003).
Actin is in cells, both in filamentous form (F-actin) and globular form (G-actin). Smooth muscle contains a substantial pool of monomers of G-actin, which polymerizes spontaneously when it is in a 8 μg/ml concentration (Pollard TD et al.2003). In differentiated vascular myocytes cytoplasmic concentration of G-actin is in this critical threshold, which requires a strict regulation from actin-binding proteins as cofilin and profiling (Kim HR et al.2003).In the smooth muscle the 80% (Zhang W et al.2008) of actin is in F form. This percentage moves to the 90% after to α-adrenergic stimuli. (Cipolla MJ et al.2002).
Actin filaments are the structural components that increase cellular rigidity and amplifies the dynamic contraction force. Also the intravascular pression,increases the actin polymerization. Adrenergic factors induce the γ-actin and to a lesser extent β actin remodeling (Kim Hr et al.2008). Transient loss of γ-actin is involved in pulmonary hypoxic neonatal hypertension (Hall SM et al.2000). In this case it was observed that the exposition to hypoxia increases polymerization of actin in myocytes contributing to the vascular rigidity and to the hypoxic vasoconstriction transmission force (Hall SM et al. 2000).
At a neonatal level myocytes polymerization in pulmonary artery, can derives from vasoconstrictor receptor stimulation that activates RhoA. Myocytes pulmonary artery presents a wide range of actin forms.

Regarding pulmonary artery's myocytes, it was demonstrated that Rho-kinase CDC42(celldivision cycle 42) activate LIMK(LIM domain kinase 1) that phosphorylates cofilin causing formation of stress fibers. Moreover, cofilin phosphorylation cuts F-actin suppressing cytoskeleton reorganization. In this case, there is a correspondence between phosphorylation of cofilin and actin polymerization in pulmonary artery (Osada-Oka M et al.2002).
Considering actin polymerization in renal arterioles in pulmonary hypoxic neonatal hypertension and like in the pulmonary artery case, it is a contractile answer to the thromboxane. KCl in renal arterioles raises actin polymerization of PPHN(persistent newborn pulmonary hypertension) in animals (Fediuk J et al.2012).
In conclusion, neonatal hypoxia increases actin polymerization in all contractile myocytes with the Rho pathway particularly important in pulmonary artery. It was speculated that wall rigidity due to polymerization can be susceptible to all down-regulation from inhibitors of kinase -Rho(Fediuk J et al. 2012); however, other ways to induce independent polymerization from Rho like in the presence of thromboxane are known (Fediuk J et al. 2012).

Changes in *Homo sapiens*: adult and senile age

Actin post-translational modifications are more evident during adulthood and senile age.
A 2007 study (Snow LM et al.2007) indicates how advanced glycation through its products AGE (advanced glycation endproducts) influences muscular protein qualities, like actin, contributing to the oldness (Fofana B et al. 2010).
The final products of advanced glycation can influence protein qualities of skeleton muscles and explain the decrease of contractility in relation with age.
The analysis (Snow LM et al. 2007) was conducted on extensor muscle along the fingers. The study was done on young rats of 8 months, old of 33 months old, and older of 36 months. Results show that muscles form older rats, present a high percentage of myofibers with AGE modified proteins. Among these there is above all actin (Snow LM et al. 2007).
These results show that AGE interact with certain skeleton proteins in old phase (Snow et al.2007).
Other recent 2008 studies (Gannon J et All 2008) indicate how phosphorylation of some amino acids in muscular proteins as actin cause sarcopenia in senile age.
In this work (Gannon J et al. 2008), in order to evidence the progress of a sacopernic muscle some muscle fibers of gastrocnemius rat muscle are analyzed. The analysis revealed that 22 muscular proteins showed a differential pattern of expression between 3 months and 30 months of analysis of fibers. Increasing phosphorylation level with age was observed in many proteins as actin. It was observed that getting old induces alterations in phosphorylated proteins that are involved in the contractile system of muscle and cytoskeleton, and mitochondrial metabolism. This confirms that sarcopenia in senile age is a complex neuromuscular pathology associated to drastic changes in structure and abundance in muscular proteins (Gannon J et al.2008).
Phosphorylation occurs in α1 actin of skeleton muscle and in α actin. Moreover post-translational modifications that include nitration of a number of skeletal muscle protein, as actin itself, are correlated with age and can be considered a significant factor responsible of decline in muscular force (Gannon J et al.2008).
In this context it is explained how post-translational modifications play a fundamental role in sacopernia and impairing rates of protein synthesis.
Recent studies indicate that some modifications of actin may be responsible for visual impairment in old age (Yu CC et al.2012).
In aging human eye oxidative stress and accumulation of oxidative pre lysosomal lipofuscin are the cause of functional decline of the retinal pigment and this contributes to age-related macular degeneration (Feeney-Burns L et al.1984). In rats presenting a specific defect of phagocytosis – RPE(retinal pigment epithelium) for lack of αvβ5, the integrin receptor develops an accumulation of

lipofuscin suggesting that the age-related blindness can also result from oxidative stress (Yu CC et al.2012).
The HNE (4-hydroxy-2-nonenal) destabilizes actin directly, and it induces destabilization of the cytoskeleton in cells RPE due to oxidative sublethal charge of these cells in vivo, associated to blindness caused by age. This can be avoided with a diet rich in antioxidants (Yu CC et al.2012).
Moreover, it is seen that other proteins with the aging are susceptible to HNE, so, both with rats and humans,it was outlined that RPE(retinal pigment epithelium) with the accumulation of lipofuscin or with the passing of years can lead to a destabilization of actin that causes damages to cytoskeleton (Yu CC et al.2012).
Oxidative modifications increase with the age and destabilize actin in RPE; it is suggested the possibility of an antioxidant diet with supplements or natural food to prevent or delay this process. A diet rich in antioxidants grape and calendula extract was tested and it was confirmed that age-related vision loss is due to the accumulation of oxidants in time (Yu CC et al.2012).
Finally, there are no specific studies on post-translational modifications in actin in the period between puberty and adulthood.

Variations related to gender: modifications in female apparatus

In some studies it was observed how in female apparatus, during both maturation of oocytes and fertilization, some modifications can appear in cytoskeleton of actin to a biochemical level which influences fertility in a number of ways.
In fact a 2013 study (Chun JT et al.2013) on actin cytoskeleton of oocytes of some star fish shows that depactin is a cofilin member ADF(actin depolymerizin factor). This is essential to prevent excessive aggregates of F-actin in cytoplasm, and it seems that depactin is responsible of Ca2+ release in the fertilization moment. Further investigations have shown how this actin-binding is necessary for vesicular exocytosis and sperm incorporation making more possible the fertilization process. (Chun JT et al. 2013).
Other studies have shown that substances such as mycotoxins and aflatoxins, that are in some food like cereal,can effect the reproductive efficiency in animals and in humans. In fact a 2014 study (HouYJ et al. 2014) reveals how the administration of contaminated corn with these mycotoxins for a four-week mice diet, to 3 different concentrations 0-15-30%, shows that the indices of germinal oocytes vesicle in ovarian animals fed in this way are strongly decreased. Instead in the embryos that cannot develop to the morula stage, the actin's percentage is reduced causing impaired nucleation of profilin 1 (Pollard Td et al.1984). Oocytes quality was examined for actin filaments and the microtubules by Hou YJ et al. 2014.
There is a decrease of these proteins that are involved in assembly, causing a real deficit (Hou YJ et al.2014). Actin low in metaphase I of oocyte after assuming this contaminated food. All this determines the destruction of the oocyte cytoskeleton, because mycotoxins interrupt cap actin formation preventing actin filaments polymerization and sequestering monomers in association with other actin-binding proteins (Pollard Td et al.1984).
Concluding, this study reveals that mycotoxins assumed in these foods compromise the quality of the oocyte and female fertilization.

Modifications in male reproductive apparatus

Similar studies carried out in male underlined how relevant some modifications found in actin are influences male fertility.
A 2007 study (Capkovà J et al. 2007) reveals that if SABP(sialic acid binding-protein), an actin-binding protein composed by 118 amino acids, is excessively present in the sperm it binds to actin preventing fundamental events like acrosome reaction and sperm motility (Capkovà J et al.2007).
This protein is responsible in male subject of oligospermia and astenozoospermia causing male infertility (Capkovà J et al. 2007).
Recent studies reveal how other actin-binding are important for male fertility.
In fact a 2010 survey (Finkelstein M et al.2010) reveals that, to acquire fertilization competence, sperm have to suffer various biochemical changes in female reproductive tract, known as capacitation and sperm capacitation requires actin polymerization, while F-actin is dispersed before the acrosome reaction.
In this study is clear how the actin-binding protein-gelsolin is inactive during capacitation and is activated before acrosome reaction (Finkelstein M et al. 2010).
The release of gelsolin is mediated by phosphatidylinositol 4,5 bisphosphate PBP10, a peptide containing PIP2-associated domain or by phospholipase C activation that hydrolyzes PIP2(phosphatidylinositol 4,5 biphosphate) and determines F-actin depolymerization during advanced acrosome reaction (Finkelstein M et al.2010).
The release of gelsolin from PIP2 with its dephosphorylation allows the gelsolin activation, provoking acrosome reaction (Finkelstein M et al. 2010).

Actin-binding protein fascin

A 2014 study (Esnakula AK et al.2014) reveals how in the females the presence of the actin-bundling protein fascin can be responsible for breast cancer or metastases.
In fact fascin (Otto JJ et al.1979) is an acting-binding protein that plays a critic role in cellular motion, because of the actin preeminent projections formation called filopodia. These have an important role in cellular migration, in migration and metastatic spread (Esnakula AK et al.2014).
Women that present this particular protein, have more predisposition to breast cancer metastasis (Esnakula AK et al.2014).

Modifications in different ethnic groups

Some studies have revealed some modifications of the actin itself or in some population with biochemical changes but only in certain ethnic groups.
A 2012 study (Bostrom MA et al.2012) revealed that ACTN4 (α actinin-4), a well- known actin-binding protein, after his mutation causes focal segmental glomerulosclerosis (GSF) in afro-american subjects. ACTN4 is expressed in various tissues, above all on glomeruli and it binds actin in cytoskeleton contributing to the structure and to the cellular motion.
Most mutations have brought an increase of actin binding of α actinin-4 with consequent formation of actin aggregation. This disease called familiar GSF is a rare dominant autosomal disease (Bostrom MA et al.2012).
A already cited survey where was noted the fascine presence, a actin-binding responsible of metastasis in breast cancer, it reveals how protein expression is identified above all in afro-american females (Esnakula et al.2014).
In fact, according to numerous surveys it was concluded that in in Afro-American females, the 85% expressed the fasciae, among arab ethnicity only the 31,0 % as represented in 8.1 figure (Esnakula AK et al.2014).
In two precedent studies of spanish ethnic females the fascine were expressed between 25,4% and 20,7% while only the 6,7% of chinese ethnic women expressed the fascine.
So is confirmed the different expression of actin-binding according the different ethnic racial groups (Esnakula AK et al. 2014).

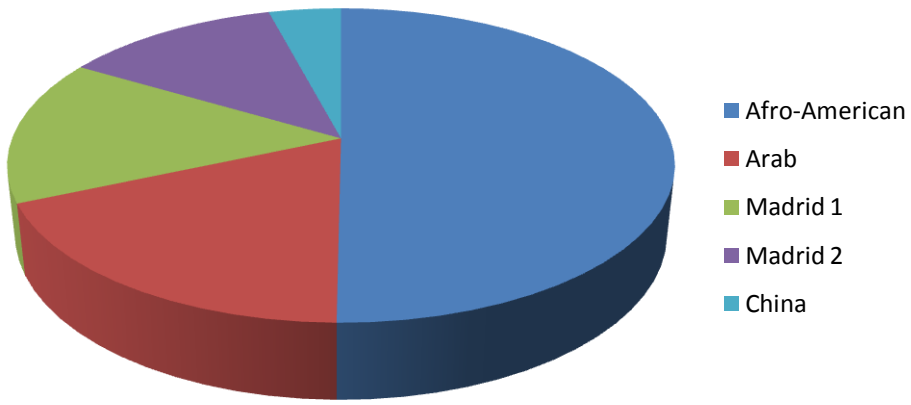


Figure 8.1 *fascine expression with incidence of invasive breast cancer in females of various populations under study.*
-Strong association of fascine expression with triple negative breast cancer and basal-like phenotype in African-American women.
Ashwini K Esnakula et All. Journal of clinical pathology, Feb. 2014

Aspects related to nutrition

It has been shown that foods can influence the health status regardless of the age. During the last 20 years, it was observed that some food components can cause post-translational modifications and can modulate the expression of proteins like actin.
For example an incorrect maternal diet alters the gene expression of the metanephros which originates the mammalian kidney; it has been observed that a low-protein diet undertaken between the conception phase and the 13th days of embryonic stage drastically reduces the glomerular number of progeny by around 20%. In cases of a low-protein diet in cofilin -1, which is a regulated actin-binding protein, a higher disassociated F-actin subunity rate is observed and this entails various urinary malformations among which renal hypoplasia. The destabilization of the cortical actin network inside the cells of the proximal tubule activates ADF with the delocalization of the apical domain (Bamburg JR et al. 1999).
Therefore, humans do need a diet rich in proteins between the conception and the 5-6th week of gestation to have a correct number of glomerules in order to develop a healthy population with no renal diseases or hypertension (Welham SJ et al. 2005).
Regarding nutrition, recently it was reported that oleuropein, a powerful anti-oxidant and anti-angiogenic agent deriving from the olive tree, can interact with the actin cytoskeleton in neoplastic cells revealing anticancer properties (Hamdi HK et al. 2005).
A study dating back to 2005 (Hamdi HK et al. 2005) confirmed how oleuropein may be an anticancer agent since it interrupts directly the actin filaments inside the cells preventing cellular proliferation. It was also demonstrated that the oral subministration of oleuropein in rats having spontaneous cancers led the cancer to decline in 9-12 days (Hamdi HK et al. 2005). This shed a light on the protective effects that a diet rich in olives has (Hamdi HK et al. 2005).
This study took into consideration adhering cancer and cells cultivated and treated for two hours with a 0.01% concentration of oleuropein, fixed and visualized with actin monoclonal antibody (Hamdi HK et al. 2005). It has been proven how this anti-oxidant can drastically interrupt the organization of actin filaments inside these cells (Hamdi HK et al. 2005).

Figure 9.1:The importance of olive

More recently, a study showed the consumption of a diet rich in anti-oxidant composed of grape, zeaxantina, calendula and lutein can prevent the oxidant stress and the accrual of lipofuscin in the human eye during senil age avoiding a functional decline in the retinal pigment thanks to the fact that this substances prevent the bound between HNE (a destabilisation of actin) and actin (Chia-Chia YU et al. 2012).

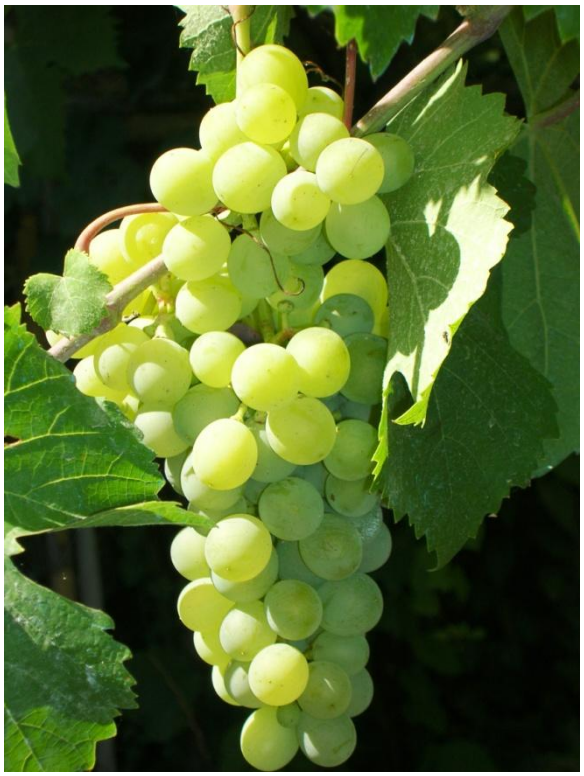


Figure 9.2*: grapes have an antioxidant power in order to prevent functional decline of the retinal pigment.*

The importance of consuming foods rich in Vitamin E in case of hypercolesterolemia has been liked to the effect playe by this vitamin on the regulation of the actin cytoskeleton inside bronchioles and alveoli (Kaga E et al. 2013).
Moroever,the assumption of harmful products like various types of mycotoxins such as deoxynivalenol, zearalenone and aflatoxins which can be found in contamined foods like cereals, seems responsible for a compromised nucleation of profillin-1, a actin-binding protein which plays an important role in female fertility. (Hou YJ et al. 2014).

Pathophysiological aspects: cardiovascular diseases

Hypoxia is caused by oxidative stress due to reactive variations to ROS oxygen and nitric oxide NO (Terman JR et al. 2013).
Nitric oxide is continually produced by nitric oxide synthase and it is necessary to keep vessel integrity (Terman JR et al. 2013).
A disequilibrium between ROS (reactive oxygen species) and NO (nitric oxide) entails cellular disfunction and cardiovascular disease. It is well known that NO and cGMP-dependent processes mediate effects that are vasodilators, and anti-proliferative. However NO-cGMP independent effects indicate a covalent addition of NO to Cys-sulfur in some proteins that play a fundamental role in the modulation of actin properties (Terman JR et al. 2013).
S-nitrosylation is fundamental to understand cellular NO mediated answers in endothelial cells in stress conditions (Terman JR et al. 2013).
S-nitrosylation of actin inhibits the effects of neutrophils integrin on β-actin. This modification was detected on residue Cys 170 after an high hypoxia exposition. Other studies revealed the importance of actin-binding like gelsolin in activating apoptosis after myocardial infarction (Li GH et al.2009).

It was observed that gelsolin association to actin is regulated by calcium and it's highly found in murine cells and in the human heart after myocardial infarction. Moreover, gelsolin is associated to cardiac insufficiency in man (Yin HL et al.1981). To clarify the gelsolin role in these pathological processes these studies used gelsolin-knockout and wild-type mice (Li GH et al.2009).
Gelsolin- knockout mice have a mortality extremely low, reduced hypertrophy, less interstitial fibrosis and good cardiac function (Li GH et al.2009).
It was also observed that gelsolin gives an important contribution to the progression of cardiac insufficiency through HIF- 1α and DNase I causing down-regulation of anti-apoptotic surviving factors (Li et al.2009), revealing a new mechanism trough wich gelsolin modulates cardiac remodeling after infract in man (Li GH et al. 2009).
Recent studies evidenced how S-glutathionylation of a protein like α actin can play a role in cardiovascular pathology (Pastore A et al. 2013).
In vitro it was shown that when occurs glutathionylation in the cardiac muscle, the α -actin polymerizes more slowly (Pastore A et al.2013).
β actin constitutes a direct target for the oxidative modification in the cardiac human muscle and its glutathionylation represents a mechanism through which glutathion modifies the sarcomere functions according to the redox state of tissues (Pastore A et al. 2013).
Glutathionylation of actin plays a limited but well defined role in contractile alteration after oxidative stress of myocardium, in particular through decrease of the actomyosin ATPase activity (Pastore A et al.2013).
Further studies evidenced that glutathionylation of G-actin delays its speed of polymerization and decreases binding cooperativity with tropomyosin contributing to the decrease of cardiac contractility during ischemia (Pastore A et al. 2013).

Pathophysiological aspects: diabetes

A British study edited in 1992 (McLean WG et al. 1992) shows that diabetic neuropathy is a common complication of secondary diabetes mellitus, although its pathogenesis remains uncertain (McLean WG et al. 1992).
The disease occurs with changes of axonal transport, which depends largely on the cytoskeleton of the axon. In diabetic rats and mice, it is observed a slowdown in axonal transport structure (McLean WG et al.1992) linked to glycation, consisting in a binding of glucose to a Lys residue in cytoskeletal like actin (McLean WG et al. 1992).
Another post- translational modification altered in the diabetic pathology is the phosphorylation of neurofilament proteins. The insulin-dependent actin glycation takes place at the level of the peripheral nerves and modifies the interaction of actin with other proteins, influencing the cytoskeleton role on axonal transport and causing alteration of neural morphology typical of the diabetic neuropathies (McLean WG et al. 1992).
A study conducted in 1993 (Pekiner C et al.1993) clarifies that the glycation of proteins such as actin in diabetic subjects, causes functionality nervous problems which drastically slow the axonal transport (Pekiner C et al. 1993). Glycation involves deformability of granulocytes containing actin filaments useful for movement and cell interactions.

Pathophysiological aspects: kidney diseases

A disorganization of the actin can cause several problems at a renal level too, especially if the consequences of an ischemia have to be faced.
The usual epithelial function depends on the integrity of cytoskeleton matrices of actin. In the kidney interruption of these matrices at the level of the renal tubular epithelial cells is believed to be a mediator of acute renal failure and ischemic heart disease (Shelden EA et al. 2002).

During ATP depletion F-actin filaments in epithelial cells increase although the reasons remain unknown. The assembly of actin in cell-cell adhesion sites could be a protection mechanism or a pathology consequence deriving from the ischemic disease (Shelden EA et al. 2002).
Was demonstrated that in cases of ATP depletion, an overexpression of HSP 27 appears to be useful in order to ensure greater stability to the filaments of actin in damaged cells (Van Why SK et al. 2003).
The first consequence of ATP depletion appears to be the loss of actin from the lamellar structures (Shelden EA et al. 2002).
In this analysis it was shown that the changes of actin are independent from the extracellular pH when there is a depletion of ATP. Also, this study found out that polymers of actin are contained within the cytoplasm, however it does not specify whether the assembly of actin filaments in polymers in the absence of ATP occurs with a normal mechanism of assembly (Shelden EA et al. 2002).

Pathophysiological aspects: infertility

As previously mentioned, the presence of actin-binding proteins like SABP provokes the suppression of the sperm motility and causes serious acrosome damages (Capkova J 2007). Similarly, gelsolin intervenes differently according to the various phases of sperm maturation (Finkelstein M et al. 2010) and depactin intervenes in the oocytes and is responsible of an adequate release of Ca2+ that stimulates a correct release of mature oocytes (Chun JT et al. 2013). This is the demonstration of how many actin-binding proteins can modulate actin cytoskeleton and consequently influence fertility and the possibility of conception.

OSTEOCALCIN

Biochemical properties and physiological role

The bone strength in vertebrates mainly derives from the hydroxiapatite. The production of this hexagon-shaped mineral is guaranteed by the presence of the osteocalcin protein (Hauschka P et al.1989).
The osteocalcin in its active form is constituted of 49 amino acids and weighs 5.8 kDa. This protein appears to be highly conserved, which means that the amino-acid sequence of osteocalcin is almost identical for all vertebrates (Hauschka PV et al.1982).
The transcription of osteocalcin is regulated by 1, 25- dihydroxy - vitamin D3, and this is one of the reasons why vitamin D is essential for bone health (Lian J et al.1989) .
The OC is initially translated as pre-pro-osteocalcin 98 amino acids long. This property comprises three parts: a protein signal composed of 23 residues cleaved following translation, a pro-peptide consisting of 26 residues and a mature protein composed by 49 amino acids (Neve A et al. 2013) .
To activate the protein, vitamin K is necessary. This vitamin is very well known because of its essential role in blood clotting. In addition, vitamin K is an essential cofactor in relation to the γ - carboxylation. The glutamyl residues of osteocalcin located at positions 17-21-24, have a second carboxyl group (COOH) added to form the residual γ-carbossiglutamic residues (GLA).
(Hauschka PV,1986). The processes described above modify the structure and stabilize the osteocalcin in a α – helix structure (Hauschka PV,1986).
The protein reaches its highest stability when it meets the Ca2 + ions folding in order to accomplish its function (Atkinson RA et al.1995). The two osteocalcin carboxyl groups on residues γ - carboxiglutamic are chelated by Ca2+ ions as demonstrated in spectroscopy, confirming that there would be no spectral change in the OC molecule following the addition of calcium ions without the intervention of vitamin K (Atkinson RA et al.1995).
More information about the osteocalcin come to us from some studies performed on the swine osteocalcin. The crystal structure of porcine OC was discovered by using a X-ray diffraction method called anomalous dispersion single iterative (Hoang QQ et al. 2003).
It has been observed that the active site of the protein has a region negatively charged that binds Ca2+ positively charged (Hoang QQ et al. 2003). Five Ca2 + ions are coordinated by three special Gla residues and an Asp in position 30. The five calcium ions are bound in order to allow to osteocalcin to anchor one additional another Ca2 + ion, so as to allow the bone to grow appropriately (Hoang QQ et al.2003).
The OC, in order to perform such process, must be able to rely on a well-defined sequential arrangement of its amino acids (Lee TY et al.2011). For example, there must be a tight core involving the following hydrophobic residues: Leu16 - Leu32 - Phe38- Ala41 - Tyr42 - Phe45 - Tyr46. Hydrogen bonds are also important in stabilizing the connection between the different α – helices, Asn 26 in the propeller α1 - α2 and Tyr 46 in α - helix 3 the helices α1 and α2 give rise to a V-shaped arrangement which is stabilized by a disulfide bridge between Cys 23 and Cys 29 (Hauscka PV et al.1982).
The structure of the protein appears to have an irreducible complexity in order to give rise to its operation. In fact, the evolutionists have not been yet able to present evidences on how the transition of the protein from invertebrates to vertebrates occurred (Laizé V et al.2005).
Furthermore, the osteocalcin function is due to the presence of 3 Gla residues (Atkinson RA et al. 1995).
X-ray studies define the molecule containing three helical regions of a C-terminal hydrophobic core and a structured N-terminal (Malashkevich VN et al.2013). All three Gla residues are located in the first helical region and interact with calcium. The C -terminal extends outward and becomes accessible to neighboring cells as the endogenous proteases (Hoang QQ et al. 2003).

Other studies have shown that osteocalcin affects the growth and maturation of the mineral phase of bone (Gundberg CM et al. 2012).
The C -terminal peptides of this protein have chemotactic activity, are precursors of osteoclasts and remodel the bone structure. In addition, it has been proven that the osteocalcin distribution in human osteons varies based on genders and age groups whilst local reductions of osteocalcin in bones are associated to a reduced cortical remodelling (Gundberg CM et al. 2012)

Clinical usefulness of osteocalcin determination

The concentration of osteocalcin changes rapidly in situations affected by bone turnover or pathological situations, as shown in figure 11.1. For example,during treatment of teriparatide, osteocalcin, increases rapidly until the end of the first month of treatment. Conversely, OC decreases dramatically in patients being treated with high doses of intravenous methylprednisolone (Peretz A et al.1996).
Osteocalcin cannot be considered a true marker of bone formation, since once synthesized this protein is secreted, in part, into the blood flow and incorporated into the bone matrix. In turn, OC is released from the matrix during the bone reabsorption. The fragments of OC that will arise contribute to increase the concentration of a free protein in the blood. Therefore, serum osteocalcin is fairly considered as a marker of bone turnover rather than a marker of bone formation (Ivaska KK et al. 2004).

Figure 11.1 *Table indicates the increase or decrease in the values of osteocalcin due to certain diseases.*

Diseases	***Osteocalcin concentration in serum***
Metabolic bone disease	↑
Osteoporosis	↑
Hypoparathyroidism	↓
Hypothyroidism	↓
Hyperparathyroidism	↑
Tyrothoxicosis	↑
Estrogen therapy	↓
Renal osteodystrophy	↑
Bone fractures	↑
Treatment with calcitonin,bi-phosphates and glucorticoid	↓
Acromegaly	↑
Bone metastases	↑
Hormone deficiency during growth	↓

Measurement of osteocalcin

Since the first method implemented in 1980 by Price and Nashimoto, other several methods have been developed to determine the presence of osteocalcin in humans (Price PA et al.1980).

Immunometric methods using monoclonal or polyclonal antibodies directed towards different epitopes of osteocalcin have frequently been used. These antibodies are then calibrated on protein extracts of human or bovine origin (Lee AJ et al. 2000).
Most methods are based on enzymatic systems, with a both competitive and double antibody format to create a "sandwich" applied on automated instruments. In other cases radioimmunometric systems are used (Rosenquist C et al. 1995).
ELISA is one of the two most common methods and consists in an enzyme immunoassay kit, where generally the microplate provided is pre- incubated with a specific antibody for osteocalcin. Afterwards, the samples are added in the various wells of the micro titration plate with a specific biotin antibody conjugated for osteocalcin.
Subsequently, the avidin conjugated with horseradish peroxidase (HRP) is added into each microtiter plate. At this point, the TMB substrate is added only into the wells containing OC that reacting with the antibody and conjugated enzyme gives rise to a change in coloration.
The enzyme-substrate reaction is then interrupted by the addition of sulfuric acid and the color change is measured spectrophotometrically at a λ of 450 nm ± 10 nm. Therefore, the concentration of osteocalcin in the samples is determined by comparing the OD of the samples to the standard curve (Monaghan DA et al.1993).
Another frequently used method for the dosing of osteocalcin is the radioimmunoassay (RIA) based on a single incubation in tubes coated with a human-osteocalcin mouse antibody (fragment 20-38). The calibrators,controls and samples are incubated for 2 hours with the radioactive tracer ^{125}I-osteocalcin in coated tubes. During this incubation, the radioactive tracer and the osteocalcin contained in the calibrators and samples compete for binding to the antibody sites on the tube. Later on, after the aspiration and washing processes, the remaining fraction bound in the tubes is inversely proportional to the radioactivity which is measured through a gamma counter. The values are calculated from a calibration curve traced with specific calibrators (Johansen JS et al.1987) .
In literature, the results of the determinations of osteocalcin obtained by applying the two different methods have often been compared. This proves the impossibility of comparing the data obtained as it is clearly shown in figure 11.2. These inconsistencies in the results are mainly due to the fact that in the circulation there are different fragments of osteocalcin of various sizes. Moreover, as noted, there are differences in the calibrators provided by each manufacturer. Indeed, these calibrators can be either of bovine or human origin. However, the two types of OC differ only in 2 out of 49 amino acids (Nielsen PK et al. 1997) .
It is believed that the discrepancies found when comparing different methods could be attributed to the ‘heterogeneity of the antibodies used and that of the molecules. Often the antibodies used have a different specificity and affinity, respect to the various fragments or the intact protein (Deftos LJ et al. 1992).
Hence, it seems that the methods that measure both the intact molecule and the fragment 1-43 provide a more realistic picture concerning the synthesis of osteocalcin (Rosenquist C et al. 1995).
Osteocalcin can be determined both on serum and plasma. However, more attention has to be paid when the measurement is made on plasma. In fact, the EDTA used as anticoagulant chelating calcium which interferes with some methods resulting in conformational alterations of the protein, with masking of antigenic epitopes. In other cases the osteocalcin loses its secondary structures making the protein susceptible to proteolysis leading to fragmentation. The lipemic samples should be avoided since the osteocalcin binds to immunologically non-reactive lipids. Hemolyzed specimen should be avoided as well since erythrocytes often release hydrolytic factors that may degrade and distort the results (Levy RJ et al.1986).
The osteocalcin molecule is very unstable in vitro. In fact, it is rapidly degraded by some proteolytic enzymes present in the blood sample. For this reason, if methods recognizing the entire molecule are used, it is strongly recommended to keep the sample at 4° C. Immediately after the withdrawal of blood, the serum or plasma must be separated from the cell. Finally, samples are stored at -20˚C

for analysis running during one month. Samples are stored at -70˚C in cases of need (Banfi G et al.1994, Garnero P et al. 1994 and Blumsohn A et al.1995) .
With regard to the interpretation of the data obtained, the sources of variability arising from the preanalytical phase which is in turn conditioned by the preparation phase of the patient must be taken into account, as well as the withdrawal and treatment method of the biological sample and the state of preservation of the sample itself.
To have true and consistent results on the measurement of OC it is useful to consider the type of immunoassay that can be either immunometric or competitive, the type of antibodies that can be either polyclonal or monoclonal, the fragment recognized by the antibody and the type calibrator used (Nielsen PK et al.1997).
The osteocalcin assay is performed to predict the risk of fracture and bone loss and to determine the status of male fertility, prevent and monitor various diseases such as cardiovascular, renal or diabetic and to see a certain lifestyle or nutritional status. Initially, the undercarboxylated osteocalcin (ucOC) was measured indirectly through the binding assay for hydroxyapatite (HAP). Only in 1997 a test that could identify the 'ucOC was finally adopted. It is an ELISA essay using standard non- OC carboxylated recombinant human and two monoclonal antibodies, where one of these antibodies recognize the fragment 14-30 Glu of a synthetic peptide. On that occasion, this assay was used to predict the risk of hip fracture in older women (Vergnaud P et al.1997).
More recently it has been adopted a particular kit ELISA able to determine in a quantitative way the ucOC . The EIA kit is an enzyme immunoassay in vitro that displays the amount of Glu -OC in serum, plasma, urine, extracts of cultured cells, supernatants deriving from cell cultures and other biological fluids.
It is the analysis of a solid phase of OC human through an ELISA sandwich with the use of 2 mouse monoclonal antibody Glu –OC. The first antibody is incubated on the plate, while the second one is labeled with peroxidase in order to bind and identify ucOC.
This assay is composed of two phases: in the first phase, the samples are coated with the antibody in a microplate. In the second one, the plate is washed and incubated with the second antibody labeled with peroxidase Glu-OC. Later, a substrate is added so that the reaction between peroxidase and substrate can occur (3,3',5,5'-tetramethylbenzedine/H2O2) and determine the final color.
The quantity of Glu -OC is determined by measuring the absorbance with an EIA(enzyme immune assay) plate reader. Accurate concentrations of the Glu –OC sample can be determined by comparing the specific absorbance with the on obtained by means of standard curves.
The serum concentration of ucOC in the male subject is 2.46 µg/L while in females 2.34 µg/L (Nimptsch K et al. 2007).

Figure 11.2 *Table refers to inconsistencies in the dosing differences osteocalcin performed by ELISA and RIA*

OSTEOCALCIN	*ELISA METHOD*	*RIA METHOD*
Healty men	*9,6 – 40,8 µg/L*	*3,0-13,0 µg/L*
Healty women	*8,4-33,9 µg/L*	*0,4-8,2 µg/L*
Post-menopausal women	*12,8-55,0µg/L*	*1,5-11,0 µg/L*

Primary structure

Osteocalcin is a non-collagenous protein present inside the bone whose concentration in serum is tied to the bone metabolism. It is regarded as an excellent biomarker for the clinical evaluation of bone diseases (Hoang QQ et al.2003).
Osteocalcin influences the bone mineralization through its ability to bind with a very high affinity to hydroxyapatite one of the most important bone mineral constituents (Hoang QQ et al.2003). Besides the bond with hydroxyapatite, osteocalcin, at a cellular level, works as a detector and recruiter of osteoblasts and osteoclasts. Such function is of fundamental importance in the process of reabsorption and bone deposition (Hoang QQ et al.2003).
Moreover, it is well known that only 10-30 % of osteocalcin which is synthesized by osteoblasts is then released into circulation. This protein is involved in blood clotting, homeostasis and transport of calcium (Kidd PM, 2010). Osteocalcin, before undergoing the modification required for its operation, called crosslinking, is composed of 100 aa with a PM of 13.500 dalton.
Its isoelectric point is between 4.0-4.5 (Galli M et al. 1984) .
The concentrations of osteocalcin are influenced by various factors such as gender differences. Indeed, it is well known that man hold a slightly higer percentage concentration of osteocalcin than women, from the age difference where the post/menopause is reached in female subjects there is a substantial rise of the protein (Galli M et al.1984). Finally, it also depends on the diurnal variation where the concentrations of osteocalcin rises at different times of the day (Magnusson P et al.1995 and Rauchenzauner M et al. 2007).
As for the primary structure in 1982 (Hauschka PV et al.1982), the OC(osteocalcin) structure belonging to apes was identified and in 1984 the OC structure belonging to cats was characterized too (Shimomura H et al.1984). In 1980, the primary structure of osteocalcin was identified in Homo sapiens as it is clearly visible in the figure 12.1 (Price PA et al.1980). In 1991 the OC structure belonging to rabbits was detected. (Virdi AS et al.1991).

Figure 12.1 *Primary structure of osteocalcin from Homo sapiens(human) 100 aa* (Price PA et al. 1980).

```
        10          20          30          40          50          60
MRALTLLALL ALAALCIAGQ AGAKPSGAES SKGAAFVSKQ EGSEVVKRPR RYLYQWLGAP

        70          80          90         100
VPYPDPLEPR REVCELNPDC DELADHIGFQ EAYRRFYGPV
```

Secondary structure

Figure 12.2 Osteocalcin α-helix structure by means of NMR and X-ray crystallography, consisting of a globular structure formed by three α-helices, a C-terminal hydrophobic core and a N-terminal destructure.

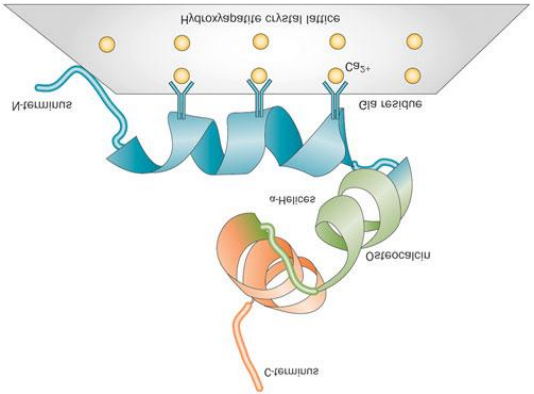


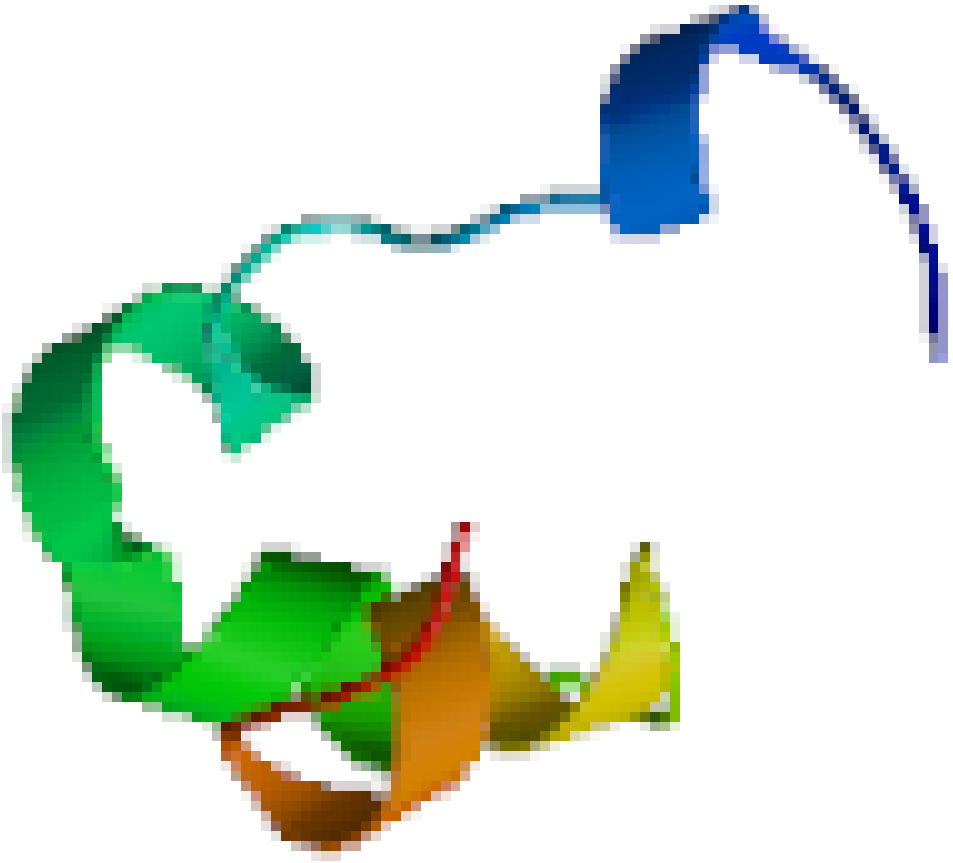
Image taken from -Hauschka PV and Carr SA Calcium-dependent alpha-helical structure osteocalcin Biochemistry 1982,21(10)2538-4

The secondary structure of OC was identified in 1982. The molecule is composed for its 40% of α - helix and for the remaining part has a β – sheet structure with the presence of a hydrophobic core (Hauschka PV et al 1982)

Tertiary structure

In 2003, the tertiary structure of osteocalcin in pigs was x-rayed (Hoang QQ et al. 2003).

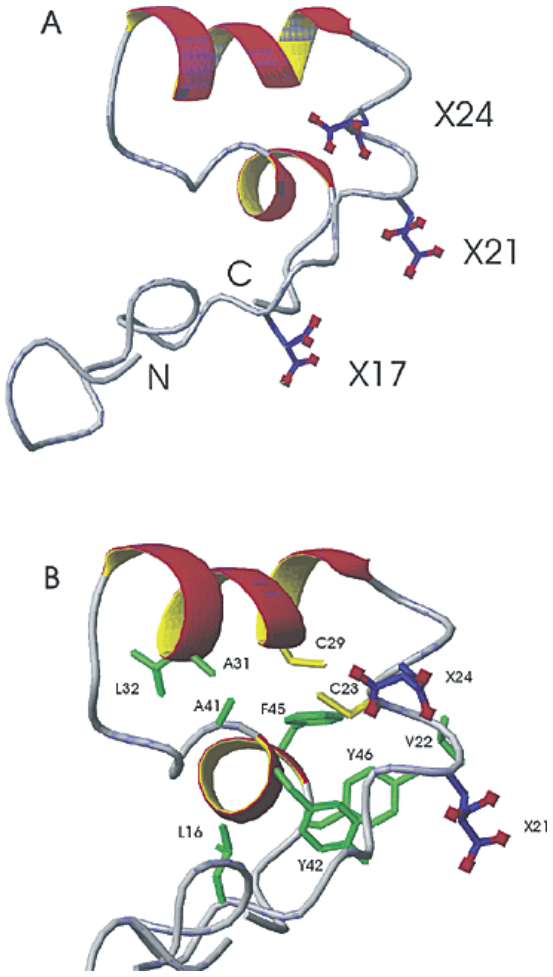
Figure 12.3 shows the tertiary structure of porcine osteocalcin.



Hoang QQ,Sicheri F,Howard AJ and Yang DS
Bone recognition mechanism of porcine osteocalcin from crystal structure-
Nature
2003, 425:977-80

Another similar study examined a solution of bovine osteocalcin titrated with Ca2 + to determine the amount of calcium to induce the maximum conformational change of the molecule (Dowd TL et al. 2003).
The picture shows some sample spectra of the 1D 1H NMR titration of bovine osteocalcin (1.0 mM) with Ca2 + in the presence of 20 mM NaCl. In both resonances significant conformational changes are highlighted (Dowd TL et al. 2003).

Figure 12.4 *Tertiary structure of bovine osteocalcin.*

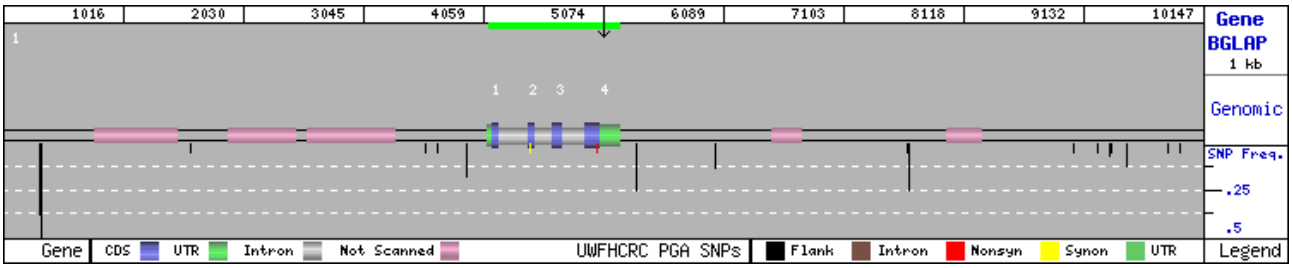


Dowd TL,Rosen IF and Gundberg CM
The three-dimensional structure of bovine calcium ion-bound osteocalcin using 1H NMR spectroscopy
Biochemistry
2003,42(25)7769-79.

Genes and expression of osteocalcin

In humans, osteocalcin is encoded by the gene BGLAP(bone gamma-carboxyglutamic acid protein). This gene is located onto the chromosome 1 as can be noticed in figure 13.1 and its receptor is GPRC6A(G protein-coupled receptor family C group member A) (Celeste AJ et al.1986).
The locus has been identified 1q25 - q31 (Puchacz E et al.1989).

Figure 13.1 *picture representing the human chromosome 1 containing the gene BGLAP*



Trascriptional gene expression is regulated by vitamin D in the promoter upstream from 1,25-dihydroxyvitamin (Staal A et al.1996) .
Osteocalcin regulates the expression of osteoblasts, acting as a negative regulator of formation (Kim SH et al. 2007), and several factors seem to modulate the expression of the gene BGLAP. Among these, there are growth factors, hormones and cytokines (Villafàn- Bernal JR et al. 2011).
All this happens thanks to the signal transduction or because of the interaction of nuclear transcription factors with the promoter of the gene in question (Villafàn - Bernal JR et al. 2011).
The BGLAP human gene is a DNA sequence of about 1700 bp (Pan LC et al. 1985).
This sequence shares a high degree of homology with various mammals such as the mouse, rat, monkey pig (Laizé V et al. 2005).
The upstream sequences associated to the BGLAP gene are about 700 bp and are organized into sequences of DNA largely preserved, sensitive to hormones binding to specific transcription factors. Downstream, however, only 4 exons and 3 introns are contained for an overall length of approximately 1000 bp (Celeste AJ et al. 1986).
The gene encoding osteocalcin is expressed efficiently in osteoblasts and odontoblasts and more weakly in the ovaries, prostate, testes, skeletal muscle, thyroid and other tissues (Jung C et al. 2001). The transcription of the gene in question is temporarily adjusted depending on the development stage of the osteogenesis and osteoblast precursor. The BGLAP is off during osteoblast proliferation but it is activated during differentiation (Lian JB et al. 1985) . The activation of the structural BGLAP gene seems to be involved in acetylation of histones and histone acetyltransferase by the simultaneous inactivation of histone deacetylase since the acetylation of the OC locus has been observed during the bone-specific OC expression and interaction of histone deacetylase with RUNX2(runt-related transcription factor 2) was designed to suppress the OC promoter (Guar T et al. 2005).
Once verified the gene activation, the transcription begins through the tie of specific elements with certain factors including TFIID, TFIIA, TFIIB, TFIIF, TFIIE and TFIIH as you can see in figure 13.2.
The most important activator of this gene appears to be VD (Blanco JC et al. 1995).
The BGLAP is repressed by transcription factors such as DLX3 (distal-less homeobox 3) , DLX5 (distal-less homeobox 5) , MEF(myocite enhancer factor-2) , MSX2 (msh homeobox-2) and the glucocorticoid-glucocorticoid complex receptor (Duverger O et al. 2013) , the most important repressor of the osteocalcin gene is RUNX2 (Hoffmann HM et al. 1994) .

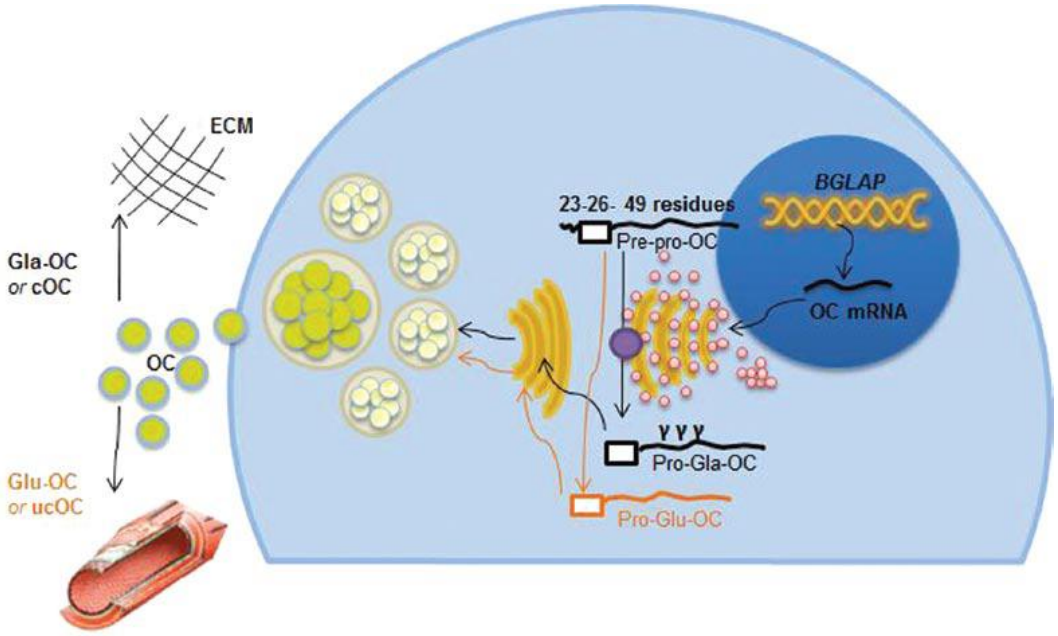
The expression of OC is regulated in a different manner by some factors listed above. For example, physical stimuli modulate the expression of OC through Wnt / β -catenin and cellular matrix (Baron R et al. 2007). Vibrations and load seem to influence the gene expression of OC (Tanaka SM et al. 2003). Sometimes, even the activation of the cytoskeleton and intracellular proteins such as actin, talin, vinculin, the paxilin and MAPK influence on gene expression of BGLAP (Franceschi RT et al. 1999). Instead, with regard to the growth factors, TGF - β maintains the gene inactive during the phase osteogenesis, while the BMP -7(bone morphogenetic protein 7) induces differentiation and expression of OC during the mineralization osteogenic phase (Maliakal JC et al. 1994). However, PTH, the calcitonin and leptin are the hormones with the greatest impact on the gene (Ducy P et al. 2000 and Hinoi E et al. 2008).

In fact, PTH and calcitonin increase the expression of OC unlike the leptin. All this occurs because of the interaction between a gene promoter and any transcription factors (Jenis LG et al.1994). Some hormones such as lipid VD, the glucorticoid, estrogen, progesterone and retinoic acid are involved in the expression of the gene in the same way BGLAP (Notoya K et al.1999 and Morabito N et al.2002) .

Moreover,some drugs are known to regulate the expression of OC , for example OPD(O-phenylenediamine) increase the release of OC through the opening of voltage-dependent Ca2 + channels in the cell line ROS(reactive oxygen species) . The nifedipine and verapamil behave as antagonists abolishing the Ca2 + effect. Other drugs decrease the levels of OC in serum (Villafàn – Bernal JR et al. 2011).

A thiazide diuretic such as hydrochlorothiazide decreases the serum concentration of OC through inhibition of BGLAP by the transcription factor c –fos. This involves a negative interaction on β - pancreatic cells causing imbalances in metabolism and promoting the rise of diabetes even in healthy subjects (Villafàn-Bernal JR et al. 2011

Figure 13.2 *image related to the transcription and translation of osteocalcin- taken from: Molecular modulation of osteocalcin and its relevance in diabetes.*
(Villafàn – Bernal JR et al. 2011)



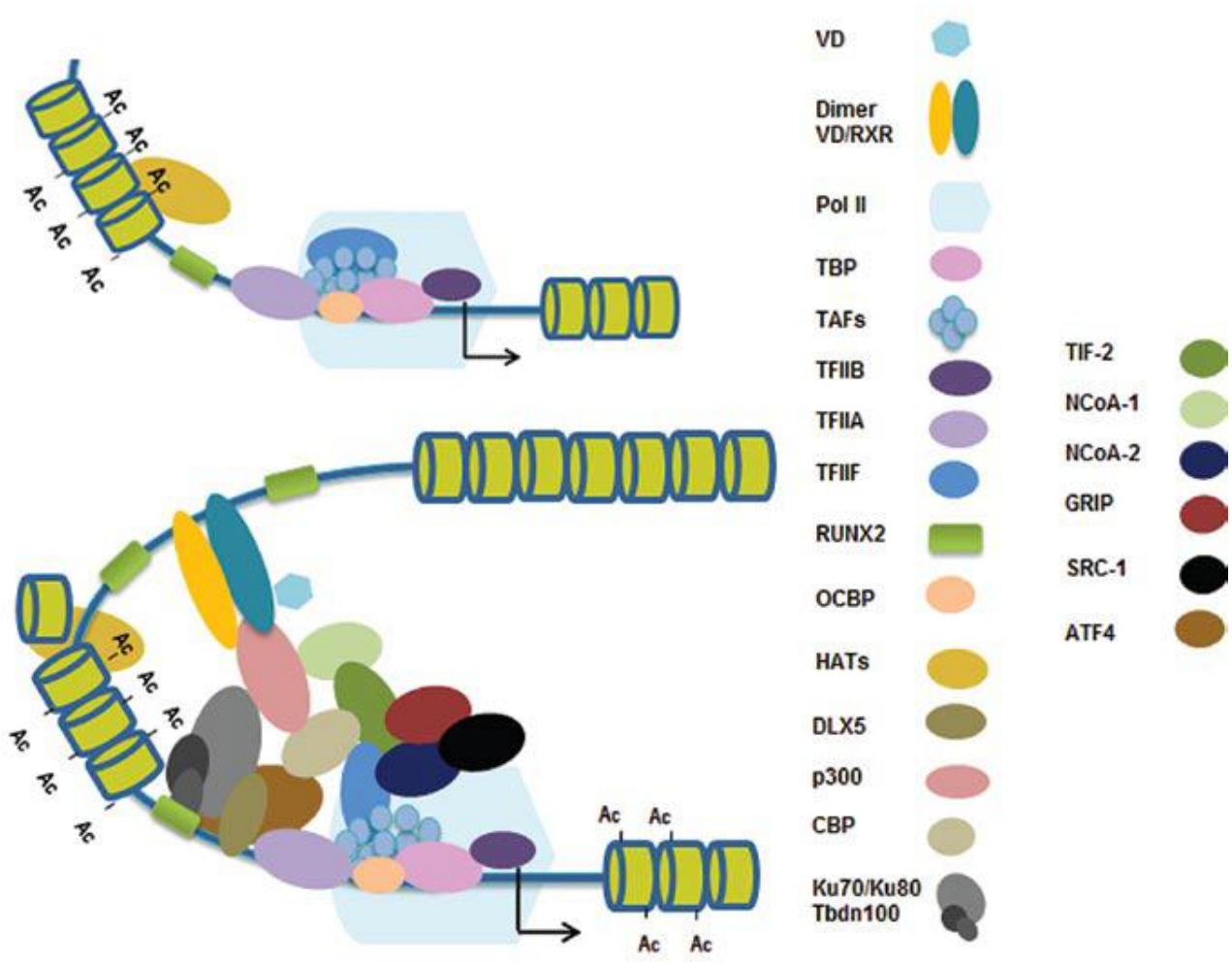


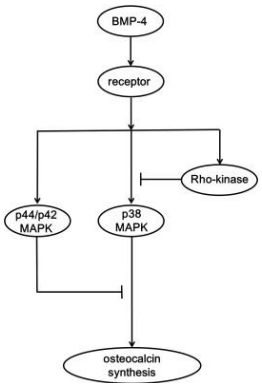
Figure 13.3 *the figure shows the main osteocalcin transcription factors and their action- Image taken from: Molecular modulation of osteocalcin and its relevance in diabetes. (Villafàn-Bernal JR et al. 2011).*

Synthesis and maturation

According to a study carried out in 2011, as mentioned earlier, the BGLAP is inactive during the osteoblast proliferation but it is activated during differentiation.
During this phase, the transcriptional activation leads to a basal transcription of the BGLAP gene, and by adding vitamin D, the gene transcription increases by 10-20 times (Villafàn -Bernal JR et al. 2011).
Acetylation at the OC locus was observed during the expression of bone-specific OC, and the interaction of histone deacetylase 3 with RUNX2 is intended to repress the promoter of OC (Hoffman HM et al. 1994).
Once that the activation of the gene in question occurs, the transcript can be improved by co-activators such as vitamin D, a receptor that increases the transcription by binding to an enhancer sequence located in the promoter of the gene distal (Frenkel B et al.1996).
The BGLAP is repressed by DLX3, MSX2, DLX5, MEF and the glucocorticoid -glucocorticoid receptor complex (Hoffman HM et al.1994) .
There are six splice variants of OC which have a distinct combination of introns (Jung C et al. 2001). These six variants encode proteins of 57-100 aa that are identical to the pre -pro -OC in the N-terminal domain. The synthesis of human OC occurs after the gene is transcribed and processed for OC mRNA, which is then translated into the rough endoplasmic reticulum.

The Pre -pro -OC is a precursor protein consisting of 98 aa (Price PA et al.1980) .
Later, in order to form a pre- peptide (23 aa) and a pro -OC (75 aa) (Price PA et al.1980) , the following steps are required. The pre- pro- peptide directs OC to the RER membrane where γ-glutamyl carboxylase (GGCX), after the carboxylated glutamic acid residues at positions 17,21 and 24 , gives rise to the formation of Gla - residues in a vitamin K-dependent process (Price PA et al. 1980) .
The Pro-OC is, finally, subjected to a final proteolytic process that originates a a pro-peptide of 26 aa and COC(carboxylated osteocalcin) or ucOC.
Both forms are released by osteoblasts following a process based on the activity of calcium-dependent potassium channels and calcium (Price PA et al.1980).
As mentioned above, certain drugs, thyroid and lipid hormones are highly responsible for the transcription of the osteocalcin protein (Villafàn - Bernarl JR et al.2011).
In a more recent study, published in 2012 (Gundberg CM et al. 2012), it is described how the osteocalcin is synthesized exclusively in the matrix and, as can be later observed, in crystallography. The molecule, in addition to performing its function thanks to the presence of the three Gla residues, has a three-dimensional structure containing three helical regions. A C- terminal region consists of a hydrophobic core and a N- terminal region unstructured (Dowd TL et al. 2003 and Hoang QQ et All. 2003). All three Gla residues are found in the first helical region and interact with calcium.
Also according to a survey, it seems that even some bone morphogenetic proteins (BMP) can stimulate the synthesis of osteocalcin (Kondo A et al.2014). BMPs are multifunctional cytokines and belong to -β transforming growth factor (TGF- β) (Kondo A et al. 2014).
The effects of BMP are exercised through the intracellular signaling of Smad proteins and protein MAP- kinase. BMP- 4 stimulates the synthesis of OC in the cells MC3T3 -E1 osteoblast -like and also the synthesis of OC is positively regulated by p38 MAP- kinase and it is negatively regulated by p44 / 42 MAP kinase as you can see in figure 13.4.
However, the exact mechanism of synthesis of OC has not yet been fully elucidated (Fukata M et al. 2001). It is generally thought that the Rho- kinase plays a crucial role in a variety of cellular functions such as smooth muscle contraction and cell motility.
Regarding the osteoblasts, it was demonstrated that the Rho and p38 MAP kinase are involved in the expression of endothelin-1-induced prostaglandin endoperoxide G / H synthase mRNA in osteoblasts. As for the BMP and the effects on bone metabolism, it has recently been shown that the inhibitor of Rho - kinase stimulates the formation of ectopic BMP -2- induced in murine cells of the skull. However, the role of Rho- kinase is yet to be entirely clear. This work was stimulated by the suppression of p38 MAP kinase in these cells (Kondo A et al. 2014).

Figure 13.4 *is represented as BMP -4 stimulates protein synthesis in osteoblasti- Image taken from: Rho -kinase limits BMP -4-stimulated osteocalcin synthesis in osteoblast regulation of the p38 MAP kinase pathway (Kondo A et al. 2014*) .



In addition, this study demonstrated that the BMP- 4- induced phosphorylation of MYPT -1(myosin phosphatase target subunit 1) in MC3T3 –E (mouse osteoblastic cell line) osteoblast cells and MYPT is a sub-unit that regulates the interaction between myosin and actin (Kondo A et al. 2014). Based on the the results obtained in this work, it became clear that Y27632 and fasudil up- regulate the expression levels of BMP-4 -induced osteocalcin mRNA.
Therefore these results support the hypothesis that Rho-kinase negatively regulates the synthesis of osteocalcin BM-4 in these cells. The importance of Rho-kinase has not been emphasized only at the level of vascular smooth muscle but also in a variety of cellular functions (Kondo A et al.2014).
Again, this study points out that OC is one of the most important non- collagenous proteins of the extracellular matrix of bone and it is expressed only by mature osteoblasts.
In conclusion, the results of this analysis show that Rho- kinase negatively regulates the synthesis of osteocalcin, BMP-4- stimulated through p38 MAP kinase in osteoblasts (Kondo A et al. 2014).

Post-translational modifications

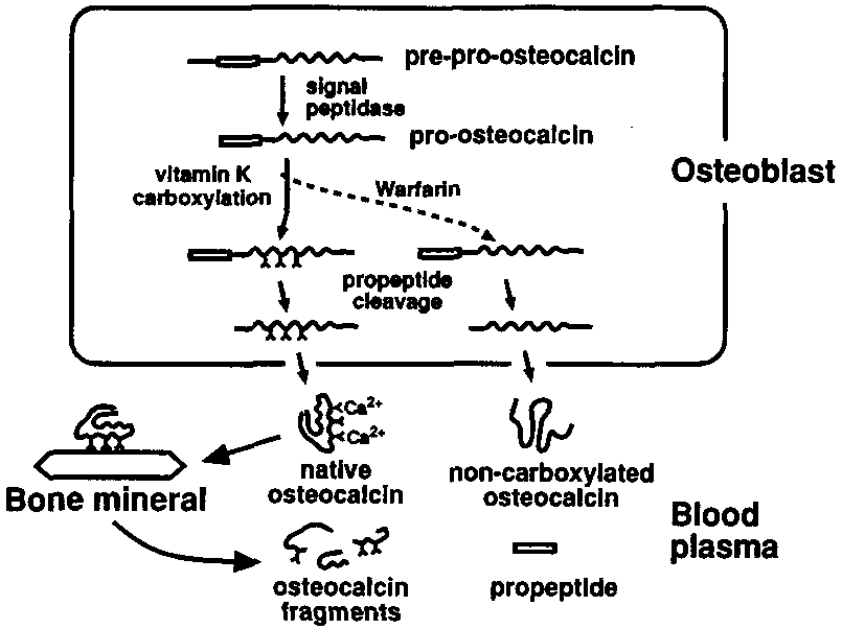
Osteocalcin, following its synthesis and maturation, undergoes some changes that are necessary for its proper workings or, as a result of specific physiological conditions.
The intracellular pro-osteocalcin undergoes changes at the level of the three GLA residues for the presence of vitamin K and for the synthesis of CO2. All this allows the α -helix conformation of the protein, thus allowing to the osteocalcin to absorb calcium ions and the hydroxyapatite bone (Hauschka PV et al.1989). As a result of this change osteocalcin undergoes carboxylation.
Human osteocalcin is located in two different states of carboxilation. Osteocalcin call COC that describes the form of fully carboxylated with all three residues carboxylates. The shape ucOC that presents no carboxylation in all three glutamic acid residues (Hauschka PV et al.1989). This form does not undergo α - helix conformation presenting lesser affinity for Ca2+ and for hydroxyapatite (Thrailkill KM et al. 2012), moreover, ucOC is present in serum at a concentration that varies between 16-21 % of the total concentration of osteocalcin.
As mentioned above , the most important and significant change in the osteocalcin is determined by the γ carboxylation of glutamic acid (GLA) residues 17,21,24 catalyzed by vitamin K.
The carboxylation is a modification of glutamate (Glu) catalyzed by gamma-glutamyl carboxylase in the lumen of the endoplasmic reticulum.
Vitamin K transforms the Glu in Gla when carbon dioxide is added in the position range in the presence of oxygen and vitamin k is reduced. The carboxylated proteins are active when bind Ca2+.
The OC comprises from 3 to 5 Gla residues to regulate bone growth and the extra -osseous calcification (Price PA et al.1981).
The most important characteristic of a site of glutamate is the uniformity around the sites of carboxylation. Another feature is the increase of Arg (the aa a positive charge) in the positions that -7-5-4-1 + 3 differ between the sites of carboxylation and non- carboxylation (Lee TY et al. 2011).
The regions -7-4 protein structures are different between carboxylated and non- carboxylated. The carboxylated proteins are classified into different groups: Gla-peptidase domain and EGF-like proteins (Lee TY et al. 2011).
The different forms of osteocalcin play a fundamental role in both physiological and pathological human organism, especially in relation to the cardiovascular and nervous system, the processes of fertility , the endocrine system and the development of the diabetic pathology .
Other studies reveal that a change, such as glycosylation of osteocalcin is a cause of bone disease in diabetes (Yan W et al. 2013). In fact the ucOC shape, presenting lower affinity for hydroxyapatite if it exceeds 50% of total OC tends to deposit in the vessels of the body by making numerous problems of pathological nature (Yan W et al. 2013) . In addition, changes in pH are responsible for

any modifications of the OC. In fact, according to a study carried out in 2012, an acidic environment in the reabsorption lacunae is always responsible for the decarboxylation of the protein in question (Schwartz V et al. 2012). Ultimately another essential modification for the proper functioning of OC is given by the already mentioned crosslinking. This change is necessary in order to have synthesis a molecule able to bind calcium and hydroxyapatite, allowing the deposition in the bone matrix.
Therefore, after the synthesis of a pre-pro-molecule with aa 98 and 11 kDa molecular weight, various post-translational modifications determined by crosslinking induce the OC to have the active form consisting of aa 49 and PM of 5.8 kDa.
A 23 peptide cue is cleaved. Three carboxyl groups are added to three of glutamate residues in the positions 17, 21 and 24, in the presence of vitamin K which is the cofactor of carboxylase. The vitamins K involved are of two types and similarly structured: 1.2 methyl 1, 4 naphtochinoni liposoluble that transforms acid glutamic residues and acid gamma- carboxyglutamic through carboxylation (Booth SL et al. 1999).
Finally, in relation to the changes the OC undergoes after its synthesis, it seems correct to recall the fundamental role of Ca2 +. Indeed it is well known that proteins such as carboxylated OC are activated only in the presence of calcium ions (Lee TY et al.2011). A further study, mentioned earlier, specifies how osteocalcin, upon the completion of post-translational modifications and once it has been expressed in its mature shape binds calcium ions. In fact 5 calcium ions are coordinated to the glutamic acid residues in each molecule of OC mediating the protein bond to the bone mineral content (Hauscka PV et al.1989).

Catabolism

Osteocalcin , after being synthesized by osteoblasts and properly modified , is largely deposited in the extracellular bone matrix (Hauschka PV et al. 1989) . In the serum OC has a short half-life of about 5 minutes, and is hydrolyzed in the kidney and liver (Taylor AK et All., 1990). For this reason the concentrations of osteocalcin are increased in patients affected by renal insufficiency. Osteocalcin exhibits a diurnal variation with a nocturnal peak that drops by 50 % the next morning (Garnero P et All., 1994).
The C- terminal is easily hydrolyzable while the N-terminal appears much more stable.
The residues 19-20 and 43-44 are susceptible to hydrolysis threefold (Garnero P et al. 1994).
It has been suggested that the residues at positions 19-20 arginil-argnil are protected from proteolysis by the incorporation of a Gla residue. This protection is given by the highly negative charge decreed by the γ -carboxylation of residues 17, 21 and 24 (Sokoll LJ et al. 1995) .
I residues 43-44 are more conserved in different species, probably due to their incorporation into the C- terminal residue (Sokoll LJ et al. 1995).
Fragments of osteocalcin were identified with the use of monoclonal antibodies in the elderly and in patients with metabolic bone disorders. Some fragments belonging to the N- terminal osteocalcin were found especially in osteoporotic patients and in patients with kidney disease (Sokoll LJ et al. 1995). The osteocalcin in its intact form is present in various individuals only to 35%. In the image below highlights the cycle of osteocalcin up to her cleavage.

Figure 15.1 *The various stages are represented in the image of osteocalcin to the catabolism of protein comes from -image Causes and Mechanisms of Linear Growth Retardation (International Dietary Energy Consultative Group - IDECG 1993, 216 pages)*



Age-related changes of OC in *Homo sapiens*

It is important to emphasize that in the different stages of growth and specifically during the elderly age, osteocalcin come across post-translational modifications both pathological and physiological. In fact, as shown in figure 16.1 it seems that the higher concentration of molecule is present in children during their rapid growth and in women having menopause. Here we expose a more adequate research that refers to a more complex analysis related to changes of OC in every phase of human life.
(Magnusson P et al. 1995 and Rauchenzauner M et al. 2007).

Figure 16.1*: Table shows the variations of osteocalcin in various age stages by the RIA method. Image taken from the "SECTION I- GENERAL CLINICAL TESTS.*

Osteocalcin	***ng/mL***	***µg/L***	***Taking drugs***
newborn	*20-40*	*[20-40]*	*Increases with anticonvulsants,calcitriol and estrogen*
Children (2-17 anni)	*2,8-41,0*	*[2,8-41,0]*	*Discreases with glucorticoid*
Healty men	*3,0-13,0*	*[3,0-13,0]*	
Healty women	*0,4-8,2*	*[0,4-8,2]*	
Post-menopausal women	*1,5-11,0*	*[1,5-11,0]*	

Variations in prenatal age

Osteocalcin in its various forms seems to have a decisive role in the early stages of fetal development in particular in regarding the skeletal and neuronal development.
In fact, an investigation dating back to 2012 (Briana DD et al. 2012) indicates how scarce values of Glu -OC and DKK -1(dickkopf-related protein 1) in uterus determine a poor skeletal development in the fetus so that widening the chance that the disease would develop during the osteoporotic age adult (Briana DD et al. 2012).
Furthermore, an american study published in 2013 (Oury F.et al. 2013) reveals that during embryonic development osteocalcin (ucOC) crosses the placenta and prevents neuronal apoptosis in the hippocampus (Oury F.et al. 2013).
In this survey, moreover, it was shown how the ' OC is important for the fetus and that the major source of this protein is of maternal origin.
This research has amply proven that the shape of undercarboxylated OC is the only one that crosses the placenta efficiently and coupled with the fact that it is also the only form of protein crossing the BBB (blood- brain barrier). That suggests that ucOC may be the more active component of osteocalcin in the brain which develops inside the fetus (Our F.et al. 2013).
In the light of these results, it is assumed that the poor bone health in the mother can affect the fetus. Decrease of OC contributes to the lack of development of certain neuronal components in the newborn. All this is due to the high incidence of psychiatric disorders in the offspring of undernourished pregnant women. However, the molecular mechanisms used by osteocalcin in the brain are yet to be discovered (F. Oury et al.2013).

Variations in neonatal age

Having established the importance of Glu- Oc in the fetus, we are now going to reiterate the importance of the various changes in the OC baby a few days after birth.
In fact, in a 2002 study (Shimuzu N et al. 2002) it is observed that a deficiency of vitamin K , during the post-natal phase, leads to a high concentration of Glu –OC, and this form is then unable to settle into the fabric bone. For this reason, the Glu -OC in the newborn enters the circulation and becomes very high compared to normal adults.
Therefore, in this investigation it was found that supplementation of vitamin K, in nursing infants, means that the γ-carboxylation occurs in a much more satisfactory manner both in the liver and in osteoblasts (Shimuzu N et al. 2002).
In this study the Authors have analyzed the changes in the urine of 18 infants from the first moments after birth (Shimuzu N et al. 2002). Although there is no difference regarding the level of OC during the first five days of the infant's life, eventually have could see the presence of Glu -OC initial state Gla -OC in the blood of infants, highlighting the low level of vitamin K in uterus and a difference of absorption capacity in the newborn of the vitamin K. Osteocalcin assumes a different ability to bind hydroxyapatite to the bone (Shimuzu N et al. 2002). In conclusion, this study showed that the majority of OC in the serum of the newborn is Glu-OC and Gla-OC turns into after five days of life, with intake of vit. K in breast milk. The same measurement of OC may be useful to assess bone metabolism and bone neonatal turnover (Shimuzu N et al. 2002).

Changes of OC in children

The OC plays a key role in the development of the child skeleton.
In fact, a work of 2008 based on a sample of 307 children reported how a higher intake of vitamin K in children during puberty leads to increased carboxylation of OC providing the child with a better quality in mass / bone (Van Summeren MJ et al. 2008).

Post-translational modifications in adults

As for the changes of OC in adulthood until the advent of senility, there have been numerous studies indicating that an adequate intake of vitamin K in the various stages of life can have a positive influence on the carboxylation of the protein in question.
In a previous investigation conducted in 2000 (Binkley NC et al. 2000) the integration of phylloquinone in healthy adults was analyzed and it was noted that a higher intake of vitamin K1 in these subjects leads to a percentage reduction of ucOC.
All this occurred in patients who had normal clotting times with a low intake of vitamin K (Binkley NC et al. 2000) .
In this study, contrarily to what had been shown in previous publications, the percentage of ucOC did not appear to be higher in post-menopausal women.
In fact it seemed that the percentage of ucOC was particularly high in young women and in young men and this reflected the low intake of vitamin K. In the group, this was crucial in to give rise to a lack of proper γ - carboxylation of osteocalcin making important physiological problems (Binkley NC et al.2000). A continuous vitamin K deficiency in adults, according to this survey, it would lead to the possible development of osteoporosis. This evidence is based on a submaximal γ - carboxylation of ucOC that is high in an associated low bone mass and an increase in osteoporotic fracture (Binkley NC et al.2000).
In conclusion, in this study it is shown that a shortage of vitamin K leads to a high bone turnover compromising the function of the calcium homeostatic system and therefore making this much needed turnover with bone loss and eventual development of osteoporotic disease. This shows that vitamin K decreases serum osteocalcin (Binkley NC et al. 2000) while the necessary concentration of vitamin K to maximize the γ-carboxylation of osteocalcin remains unknown. However, it is clear that that an extra dose is needed in cases of a high serum concentration of ucOC (Binkley NC et al. 2000).
Also, in another investigation held in Thailand in 2005 (Suntrapa S. et al. 2005), shows how ucOC is greater in older women with low bone turnover, low BMD and presenting a high risk of osteoporotic fractures.
In the survey the women show a high ucOC, due in particular to the low intake of vitamin K because of a poor non-rural diet of the examined women. (Soontrapa S. et al. 2005).
Yet another study has shown that, in 357 Thai volunteers divided into different age groups, the ucOC is found in higher concentrations in women aged between postmenopausal and senile age with respect to the band of women young people. Everything must be justified by a different intake of vitamin K and hormonal (Bunyaratavej N et al. 2005).
One korean study carried out in 2010 (Kim SM et al.2010) differs from the results of previous investigations. In fact, by using the same method of investigation consisting in splitting a group of volunteers in different age groups, a higher value of ucOC was shown in the group of women in their twenties followed by the group of the fifties. All this could simply be justified by the fact that the various groups, in this case, had followed a different nutrition with different vitamin K intakes making a difference in carboxylation' osteocalcin (Kim SM et al.2010).

It seems clear that in healthy and young adults making a healthy diet rich in vitamin K, osteocalcin appears to be adequately carboxylated so that preventing the deposition of ucOC in the vessels and preventing cardiovascular and other diseases.

Post-translational modifications in elderly

It is very useful to highlight how even in older subjects a difference carboxylation of the OC can provoke very significant differences.
In fact, the analysis of a study dating back to 2009 (Shea MK et al. 2009) highlights the various forms of osteocalcin in their multiple states of post- translational modifications. This analysis in detail refers to insulin resistance both in males and in elderly women (Shea MK et al. 2009). The various associations between serum measures and total osteocalcin have been analyzed, carboxylated and undercarboxylated osteocalcin and insulin resistance in 348 men and women of mean age 68 years, of which 58% women, all non-diabetics.
The evaluation was carried out using the homeostasis model of insulin resistance (HOMA -IR). While associations between each form of osteocalcin at baseline and 3 -y change in HOMA -IR were examined in 162 middle-aged adults 69 years where 63 % were women, all without supplementation of vitamin K (MK Shea et al. 2009). The results showed that in older adults without diabetes circulating undercarboxylated osteocalcin was not associated with insulin resistance. On the contrary, high levels of carboxylated osteocalcin and total osteocalcin were associated with insulin resistance supporting a potential link between skeletal physiology and insulin resistance in humans (Shea MK et al.2009). It must be emphasized unclear what turns out to be the role of vitamin K in this investigation (Shea MK et al. 2009).
Subsequent studies have also shown that an antioxidant as the tocotrienol can positively influence osteocalcin preventing the loss of bone mass in postmenopausal women and in those in older age (Muhammad N et al.2013) .
The examples mentioned so far show how the post-translational modifications of osteocalcin intervene depending upon age, but also that in all cases a correct integration of vitamin K is necessary.

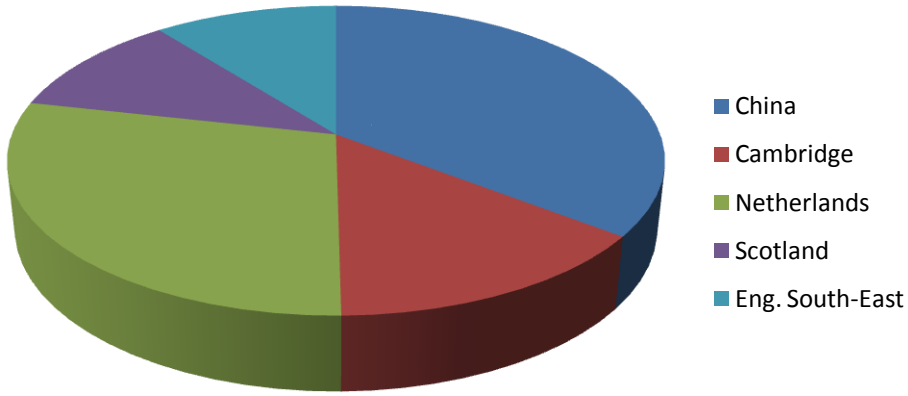
Differences in post -translational OC linked to gender

As listed above it is known that there are differences in the concentration of osteocalcin by gender. In fact, using the ELISA methodit is estimated that there is a slightly higher concentration of the molecule in males (Magnusson P et al.1995 and Rauchenzauner M et al.2007).
In fact , the shape of ucOC in the Leydig cells is coupled to the G protein , binds to the receptor GPRC6A promoting the production of testosterone and decreasing apoptosis of germ cells (Schwetz V et al. 2012). In fact, it was found that the COC appears to be associated with insulin resistance in both male and female subjects (Shea MK et al.2009). On the contrary, it is seen that the shape ucOC is increased in elderly women and in post-menopausal women. However, only the male subject is important to note that the form of ucOC brings an increase in fertility.
Other studies have shown that the OC due to the increase in Camp in the Leydig cells in both humans and mice. The OC goes to interact with the receptor coupled to protein G Gprc6a A deletion of the gene Gprc6a in males due to lower testosterone and decreased fertility. Further analysis determined that the OC binding to Gprc6a rule in CREB –dependent (Camp element binding-protein dependent) manner the expression of enzymes required for the biosynthesis of testosterone in the Leydig cells by highlighting the endocrine role of this protein (Patti A et al 2013).
Further studies published between 2012-2014 confirm the endocrine role of ucOC (Karsenty G. et al.2014) regarding the influence of this protein on the Leydig cells resulting in increased production of testosterone and greater male fertility (Oury F et al.2013) .
Thus, it is well seen as the ucOC plays different roles depending not only on age but also to the different gender.

Modifications in different ethnic groups

According to various investigations conducted during the years, it is of fundamental importance to put some hints also with regard to the differences to which the OC might encounter in H. sapiens according to different ethnicity. Indeed, various studies carried out during the years in order to determine its importance.
A first relevant study was done in 2004 (Yan L et al. 2004) bringing to light the differences in post-translational modification of osteocalcin in two populations: that China's Shenyang and the UK’s Cambridge.
In this study the Authors found that there was an abnormally high percentage (more than 50% appropriate rate) of ucOC the British population, all this according to sources reported by the analysis. It was possible because of a poor diet of vitamin K1 by the british people,due to lack of food containing such vitamin in those areas (Yan L et al. 2004).
During the same analysis it was shown that ucOC is even higher among the in habitants of Scotland. The concentration of ucOC is highest among habitants who came from the south -east of England as they had less access to products rich in vitamin K, as can be appreciated in figure 18.1. Instead the subject of the Netherlands presents a sufficient average intake of vitamin K by ensuring that the contents are the values of ucOC (Yan L et al. 2004).

Figure 18.1 *intake of daily vit. K in the various member µg/d*



In addition, as stated previously,both for Korean and Thai populations some analysis have been done to evaluate the post-translational modifications of OC in different age groups of female subjects.
A final significant study dating back to 2010 (O'Connor E et al.2010) was executed on young women from Northern Europe.
In this study we wanted to understand whether a higher intake of vitamin D (vitamin that seems to induce carboxylation of OC) resulted in improvement status carboxylation of osteocalcin preventing hip fracture. Disease is very common among teenagers in northern Europe. Unfortunately, this survey shows that increasing vitamin D does not improve the carboxylation of OC but only a greater intake of vitamin K can do that (O'Connor E et al. 2010).

Aspects related to nutrition

As already stated in this analysis, the element responsible for the majority of post-translational modifications of osteocalcin is vitamin K, in both its K1 and K2 forms, especially in regard to the process of carboxylation of the protein (Booth SL et al.1999).

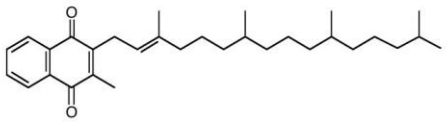


Figure 21.1 *Structure of vitamin K1.*

Vitamin K1 is assumed following the mediterranean diet and it is essential for the carboxylation and functional alterations of the OC. It is found in vegetables, green leaves, broccoli, olive oil and soybean oil.

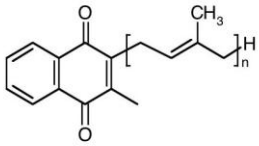


Figure 19.2 *Structure of vitamin K2*

Vitamin K2, which is useful for the operation and various modifications of the OC is assumed with a diet of cheese and dairy products. (Booth SL et al.1999).
Therefore, to make the carboxylation occur in a more appropriate manner, a balanced intake of vitamin K is required and takes place following a diet rich in certain foods.
Numerous studies over the years have shown how and what foods can have a positive influence on the changes of OC.
A relevant work of 1999 (Booth SL et al. 1999), highlights how the phylloquinone (vit. K1) is present in dark green leafy vegetables and some vegetable oils although not always in sufficient quantities for the body (Booth SL et al. 1999). The aim of this study was to compare in more or less young adults the bioavailability of vitamin K obtained from a vegetable with the addition of a fortified oil. The survey was carried out in three periods of fifteen days, with 36 examinees who took a mixed diet containing 100 µg of phylloquinone.
In the first two periods the diet to which subjects underwent was built for five days with broccoli containing 377 µg of phylloquinone daily with oil and supplemented with 417 µg daily of phylloquinone . It is valued at the end of five days the difference in vitamin K1 and the variation of the concentration of ucOC in plasma (Booth SL et al. 1999). The result shows that both the young and the elderly phylloquinone was increased and ucOC values were found to be lower (P = 0,001) after l ' intake of broccoli and olive oil supplemented (Booth SL et al. 1999).
The γ -carboxyglutamic acid in urine did not change in response to supplementation.

In summary, following this diet values phylloquinone in plasma no longer have particular differences between young and elderly contrary to the initial state where the vitamin K1 was higher in young and showed much lower levels in the elderly. Finally, in both cases ucOC percentage turns out to be much lower after the diet. Strengthens the idea of how vitamin K1 has a decisive role in a modification so important at the physiological level for a protein as osteocalcin (Booth SL et al. 1999).
However, the importance of taking vitamin K in foods was evident already from the first days of life as evidenced by a study previously cited, with 18 Japanese infants. In fact, the work mentioned above, showed how a ' addition of vitamin K in breast milk can promote within 5 days after birth the transformation of Glu-OC to Gla -OC bringing a greater bone development in the baby from the first moments after birth (Shimuzu N et al. 2002).
Later on,in subsequent studies it was decided to examine whether a diet enhanced with antioxidant - rich vitamin E might lower the percentage of ucOC in favor of a greater percentage of COC.
In the study in question, however, it is evidenced that in the subjects considered the percentages of ucOC remained unchanged not show specific differences in post-translational modifications of OC (Booth SL et al. 2004).
In the subsequent years, further investigations regarding the influence of food on the changes of OC, determined how the extra virgin olive oil may play a key role in post-translational modification of osteocalcin. Especially for what concerns the carboxylation of the protein in question.
Indeed , a work of 2011 (Bruge' F et al. 2011) pointed out the possibility to verify in particular the bioavailability of MK -7 (menaquinone 7) and its impact on the state of the carboxylation of OC .
In order to verify all the 12 healthy volunteers were instructed to take a daily dose of extra virgin olive oil alone or integrated with two different doses of MK- 7. After the plasma was analyzed and verified the levels of carboxylated and undercarboxylated osteocalcin.
It is understood that only the dose of 90 µg of MK- 7 in 20 ml of olive oil had effects on the carboxylation of OC. In practice, only at this dose ratio between ucOC and COC ranged dramatically towards a greater amount of COC bringing better health to bone and decreasing values of ucOC.
Thus, reducing the risk of vascular disease in the individual, the treatment was carried out for 2 weeks (Bruge' F et al. 2011).
A limitation of this study could have been caused by the fact that the subjects in question were all young and healthy. Further observations have been asked to prove that the oil affects the OC in different states regardless of supplemental vitamin E. From this analysis, it can be concluded that supplementation with extra virgin olive oil enriched with MK -7 can make an increase of vit. K in plasma and improve bone mineralization (Bruge' F et al.2011).
A further study that demonstrates the importance of taking olive oil for the carboxylation of OC dates back to 2012 (Fernandez -Real JM et al. 2012). The goal was to assess the effects of a diet control consisting of a low-fat or extra virgin olive oil or enriched with dried fruit to examine in the various forms of osteocalcin and bone markers in elderly men at high cardiovascular risk.
It is of our interest that in this study, the total of OC and ucOC in 127 elderly subjects was examined. The concentration was analyzed in three phases of the diet (Fernandez -Real JM et al. 2012).
The subjects all had similar characteristics.
It is seen that the concentration of COC after intake of olive oil and olives increased and decreased the ucOC in the circulation. From this observation it was concluded that the consumption of a mediterranean diet enriched with extra virgin olive oil for two years brings in the serum added quantities of osteocalcin providing greater protective effects on the shape of undercarboxylated osteocalcin (Fernandez -Real JM et al. 2012).

Figure19.3 *picture representing the olive oil for a good carboxylation of osteocalcin .*

After realizing the importance of olive oil with regard to the changes of OC, it seems also fair to mention how important the intake of palm oil is due to a fundamental element called tocotrienol (Soelaiman IN et al.2012). As mentioned previously, a study dating back to 2012 (Soelaiman IN et al. 2012) revealed how a powerful antioxidant such as tocotrienol increases bone formation by positively influencing OC (Soelaiman IN et al. 2012).

In addition, it seems that this positive influence on the assumption of palm oil, or alternatively, of products containing tocotrienol is essential for post-menopausal women since that it affects OC. Especially when we are facing a loss of bone mass due to a lack of estrogen thus reducing the rate of bone turnover in that age group appears to be high and thus ensuring better health in skeletal (Muhammad N et al. 2013).

Pathophysiological aspects: cardiovascular diseases

Finally, the analyzed studies are very important to verify how post-translational modifications of osteocalcin can affect people’s state of health.

Numerous studies show how the carboxylation of osteocalcin may affect the development of certain cardiovascular diseases.
A study dating back to 2006 (Wang Y et al. 2006) indicates that a specific polymorphism of VKORC1 , which represents the gene for vitamin K epoxide reductase complex subunit 1, is due to abnormalities in all K-dependent proteins such as OC (Wang Y et al. 2006).
In the case of the OC is not properly carboxylated bringing greater levels of ucOC. This form as already stated several times, being less akin to bond with hydroxyapatite is deposited in the plasma and becomes responsible for the development of diseases such as stroke and coronary heart disease.
The present investigation shows that polymorphisms of VKORC1 increase of almost the double of the risk of stroke, coronary artery disease and aortic dissection.
The subjects with the TT genotype show a lower activity of VKORC1 heading for a lower k - dependent carboxylation of proteins resulting in more undercarboxylated OC (Wang Y et al. 2006).
The biological activity of these proteins requires proper γ - carboxylation, in fact adequate γ – carboxylation of OC is important to decrease vascular calcification.
In addition, it was found that low levels of undercarboxylated osteocalcin and PIVKA –II(protein induced by vitamin K antagonist-II) antigens are associated with the C- allele of the VKORC1 SNP2255 that supports the idea that the association between the VKORC1 haplotype block and vascular disease is attributable to VKORC1 (Wang Y et al. 2006) .
Another more recent study has determined that in patients with coronary atherosclerosis (Okura T et al. 2010) increased circulating values of OC and endothelial progenitor cells to osteoblasts, assuming a link between vascular calcification, atherosclerosis carotid and OC.
In this survey the relationship between carotid calcification and ucOC in patients with hypertension has been taken into account (Okura T al. 2010).
Vascular calcification is an important clinical entity in cardiovascular disease and is correlated with vitamin K.
In this analysis has been demonstrated for the first time that ucOC is associated with calcification of the carotid artery in hypertensive patients and that this association is independent of the function impairment.
The patients considered in this study had an age between 60-83 years. This study also revealed that all patients analyzed had both carotid calcification of the aorta and that, on the contrary, only 60% of patients with aortic calcification had carotid calcification also.
In this study it was shown that it is highly determining the presence of the ucOC for carotid calcification, suggesting that OC is overexpressed in calcified plaques of the aorta. Even the vitamin-K dependent protein MGP (matrix Gla protein) is important for the inhibition of vascular calcification and its shape undercarboxylated is responsible for the calcification at the vascular level (Okura T et al. 2010).
Another analysis found as ucOC (Prats- Puig A et al. 2014) is responsible for the calcification of the vessel walls and pairs linked to cardiovascular risk markers in children with metabolic syndrome. In fact, the OC appears to be present in atherosclerotic plaques and its presence in the plasma can be held responsible for the calcification of the vessel walls.
In this case, it has been hypothesized that the presence of serum undercarboxylated OC would be linked to a marker of vascular risk in children with metabolic syndrome family (Ptrats -Puig A et al. 2014). The analyzed children had specific characteristics: between the ages of 5 and 10 years with no pubertal development. Children with congenital abnormalities, blood, liver, kidney anomalities and chronic diseases were not taken into consideration as well as those who had been under drugs during the previous month. None of the children falls entirely within the parameters of the metabolic syndrome (Ptrats –Puig A et al. 2014), children included had a parent and two grandparents with the metabolic syndrome with priority given to children who had the metabolic syndrome in both parents. In these children, the OC was measured by enzyme immunoassay and ucOC by solid-phase enzyme immunoassay (Ptrats – Puig A et al.2014).

The results reveal an association between ucOC have appeared in the serum and known cardiovascular risk factors in metabolic syndrome in children since prepubertal (Ptrats – Puig A et al. 2014).
The high levels of OC are associated with a higher prevalence of carotid atherosclerosis in post-menopausal women and in diabetic patients, although controversial results demonstrate that the OC contrary is associated with a smaller thickness of the arteries.
All this shows that these differences can probably be dictated by the different state of carboxylation of OC. In this case it is assumed that the shape of ucOC is the one that exerts a deleterious effect on the vessel wall of healthy children with cases of metabolic syndrome family (Ptrats-Puig A et al. 2014).
The ucOC is confirmed to be a risk marker in children with cases of metabolic syndrome family (Prats- Puig A et al. 2014).

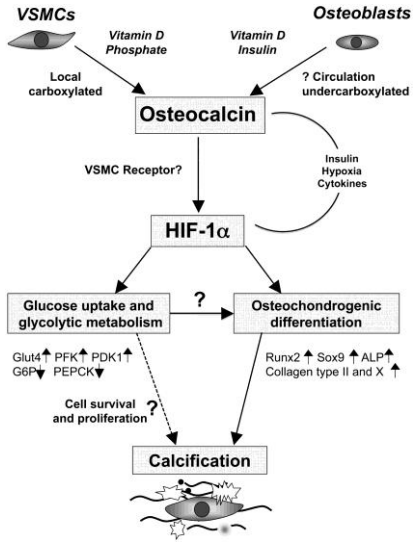
Pathophysiological aspects: diabetes

As for the changes that may have pathophysiological implications, it appears to be very important to analyze the actions of osteocalcin against β cells of the pancreas and the development of the diabetic pathology.
A first study in 2009 (Hwang YC et al.2009) determined for the first time the effects of osteocalcin in serum after amendment post- translational γ - carboxylation on β -cell proliferation. Insulin secretion and improvement towards the latter, determining the role of undercarboxylated osteocalcin in glucose metabolism in humans (Hwang YC et al. 2009) , the purpose of the analysis was to determine the association between osteocalcin undercarboxylated , the β cell function and insulin sensitivity in humans.
199 men aged 25-60 years (mean age 47 years) who had never been treated with glucose lowering agents were examined, and OGTT (oral glucose tolerance test) was performed with other metabolic parameters such as BMI(body mass index) , BP(blood pressure), lipid profile and both plasma levels of undercarboxylated and carboxylated osteocalcin were measured (Hwang YC et al. 2009).
Later, the entities in question, after being divided up into tertiles showed after some adjustments due to age and body mass, that undercarboxylated osteocalcin at higher levels has been associated with higher levels of HOMA- B%(homeostatic model assessment beta cell function) representative of the β- phone function while the upper osteocalcin tertile was associated with lower values of HOMA –IR (homeostatic model assessment –insulin resistance) that indicate resistance to insulin (Hwang YC et al.2009). High levels of both forms of carboxylated and undercarboxylated osteocalcin were associated with improved glucose tolerance. In this context, the undercarboxylated form of osteocalcin may be associated with a function β -cellulare more than adequate and the carboxylated form may be associated with improved insulin sensitivity in middle-aged male subjects (Hwang YC et al.2009).
In another study were considered to be forms of serum total OC and ucOC as markers to highlight a possible development of type 2 diabetes (Nganmukos C et al. 2012). In fact, this work is reported as the total serum OC is significantly lower in men who then develop diabetes type 2 contrary to the values of ucOC that do not vary. Sometimes a decrease in body mass and an increased intake of calcitriol increase the concentration of OC bringing greater glucose tolerance, while, the ucOC is inversely associated with the plasma glucose and fat mass in type 2 diabetes by making similarly to a better glucose tolerance and an improved β-cell function as we learn in figure 20.1 (Nganmukos C et al.2012), although this is not shown in this analysis, a report of ucOC with respect to the subsequent development of diabetes in contrast to the values of OC (Nganmukos C et al. 2012).
Furthermore, this survey showed that the factors that most influence the bone metabolism and OC are vitamins K and D, the use of bisphosphonates and glucocorticoids (Nganmukos C et al. 2012).
Another study in 2012 (Trhaikill KM et al.2012) revealed that mice without OC have a lower number of pancreatic islets and increased glucose intolerance.

In patients with type 2 diabetes it has been shown that the presence of ucOC improves glucose tolerance in middle-aged men (Trhaikill KM et al.2012), and unlike what was expected , there were substantial differences between the concentrations of ucOC in patients with type 1 diabetes and type 2 diabetes .
The studies about mices show that ucOC has a direct effect on adipose tissue inducing the production of adiponectin but not that of leptin (Trhaikill KM et al. 2012).
Leptin is negatively correlated with ucOC but only in patients with type 1 diabetes as we learn in figure 20.2.
The IGF –I (insulin-like growth -1), a homologue of insulin, plays a key role in the control of serum concentrations of OC in both types of diabetes (Trhaikill KM et al.2012).
CTX (cardiac transplantation) is correlated with OC in type 1 diabetes as well as GLA -OC, thereby supporting a role dictated by the decarboxylation of osteocalcin in bone resorption (Trhaikill KM et al. 2012). Other parameters that have shown strong associations with uc -GLA- OC and OC are: age, CTX,HbA1c IGF-1, PTH (parathyroid hormone) and 250 HD, confirming that GLA -OC and ucOC are regulated by a number of development paths and trails metabolic regardless of the state of insulin (Trhailkill KM et al. 2012).
Another very important study reveals as the ucOC shape is always very important for the regulation of energy metabolism by acting both on the pancreas that on Leydig cells (Schwetz V et al.2012). It is shown that the ucOC acts on the pancreas , the gonads, adipose tissue and causing increased secretion of insulin resulting in greater sensitivity towards it , increasing the proliferation of beta cells of the pancreas and male fertility (Schwetz V et al. 2012).

Figure 20.1

highlights ul role of OC
in the metabolism of glucose - Image taken from :
A Novel Vascular and Metabolic Osteoinductive Factor?
Kasputin AN and Shanahan CM
Arteriosclerosis,thrombosis,and vascular biology
2011,31:2169-71..



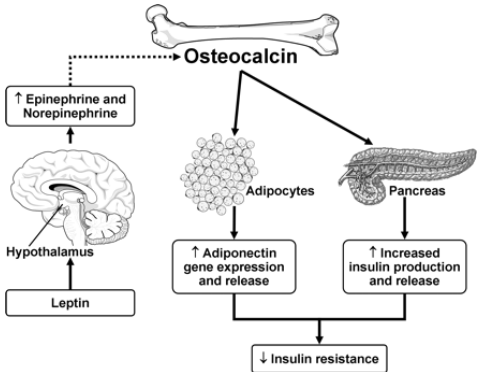


Figure 20.2 *shows the role of OC is associated with serum osteocalcin*
Serum osteocalcin is associated with measures of insulin resistance,adipokine levels,and the presence of metabolic syndrome.
Saleem U, Mosley TH Jr and Kullo IJ
Arteriosclerosis,thrombosis,and vascular biology
2010, 30: 1474-8.

Pathophysiological aspects: kidney diseases

Among the pathological aspects where the importance of post-translational modifications of OC has been detected, there are also some kidney diseases.
In fact, it is has been seen that the vitamin K deficiency is always associated with extraosseous calcification in healthy adults , and some factors such as apolipoprotein E influence the transport of vitamin K in peripheral tissues (Pilkey RM et al.2007). An analysis demonstrated that patients on dialysis who presented a particular apoE phenotype, more precisely apoE4, had a higher percentage of ucOC compared to all other phenotypes, showing that apoE4 affects the γ-carboxylation in patients with kidney disease on hemodialysis (Pilkey RM et al.2007).

Pathophysiological aspects: infertility

To summarize, the most interesting among all the pathophysiological aspects relating to post-translational modifications on osteocalcin are those related to fertility.
Specifically, we will be exclusively discussing about male fertility because, although the existence of osteocalcin in the ovarian tissue has been proven, there have been no relevant investigations.
Indeed, as already stated above, various studies that have been developed in 2012 (Schwetz V. et al. 2012) confirm the importance of ucOC form of osteocalcin, which in the Leydig cells is coupled to the G protein to go and bind with its respective receptor GPRC6A to favor a high amount of testosterone in avoiding that it has an apoptotic process as regards the germ cells (Schwetz V et al. 2012) .
That same year, the American group of Frank Oury confirms what had been expressed previously. Another study dating back to 2013 (Patti A et al. 2013) has confirmed what has been said earlier on demonstrating that a deletion of the gene Gprc6a due to lower testosterone in males and decreased fertility. Further analysis determined that the OC binding to Gprc6a rule in CREB -dependent

manner the expression of enzymes required for the biosynthesis of testosterone in the Leydig cells by highlighting the endocrine role of this protein (Patti A et al.2013).
Furthermore, after analyzing the previous studies proving that osteocalcin in its undercarboxylated shape is important for fertility and for the production of male testosterone (Schertz V et al. 2012 and Oury F et al. 2012) this study, previously analyzed for what concerns the diabetic pathology, it also demonstrates for the first time that ucOC is positively associated with the free testosterone in men with type 2 diabetes. So far, it was believed that ucOC appears to be determinant in energy metabolism as an endocrine hormones influences male fertility, but the relationship between osteocalcin and serum testosterone levels in humans had not been clarified yet.
In this new study in question, to clarify this relationship , 69 male patients with diabetes mellitus type 2 were recruited and markers of total osteocalcin were measured (TOC), the ucOC , phosphatase bone-specific alkaline,collagen type I, as some gonadotropic hormones LH and FSH(follicle-stimulating hormone) and free testosterone (Kanazawa I et al. 2013).
The analysis showed that ucOC / TOC (total osteocalcin) ratio were positively associated with FT(free testosterone) and negatively with LH regardless of age, duration of diabetes, body mass index and hemoglobin. However, neither TOC, BAP (bone alkaline phosphatase), uNTx (urinary N-telopeptide) have been associated with gonadotropic hormones or levels F. (Kanazawa I et al. 2013).
Finally, the present work indicates that ucOC is associated positively with FT and negatively with LH (luteinizing hormone) in patients with type 2 diabetes (Kanazawa I et al. 2013).
All this seems to support the evidence that ucOC is involved in the production of testosterone in male subjects (Kanazawa I et al. 2013). However, in the same year an austrian group declared, after making some inquiries at the Medical University of Graz, between 2011-2012, on male subjects suffering from infertility to have established a correlation between COC and OC with the production of testosterone and LH with ucOC, even if no type of OC confirmed the risk of oligozoospermia so that risking to not confirm the responsibility of OC in male infertility (Schwetz V et al. 2013).
Finally, a work of 2014 (Karsenty G et al. 2014) has once again confirmed the responsibility of OC in the process of fertility in male subjects. Indeed, it has been reiterated that when osteocalcin is undercarboxylated, it binds to a receptor G -coupled ,Gprc6a on the pancreatic cells by increasing insulin secretion, while, on the muscle and white adipose tissue to promote the synthesis of glucose, and still on the Leydig cells to promote the synthesis of testosterone.
It was thus demonstrated the biosynthesis of testosterone through the testis -bone- pancreas axis that is parallel and independent from the hypothalamus- pituitary- testis (Karsenty G et al. 2014).
Lastly, in order to prove the biological relevance of OC from mices to humans, it has been shown that Gprc6a is a new potential susceptibility locus for testicular failure in humans.
These findings shed new light on the importance of the endocrine role of the skeleton and provide credibility to the search for additional endocrine functions of this organ (Karsenty G et al. 2014) .
So, it is clear how the post-translational modification that transforms the OC in ucOC is fundamental in adulthood not only for energy metabolism and glucose but also with regard to the male fertility as can be appreciated in Figure 20.3.

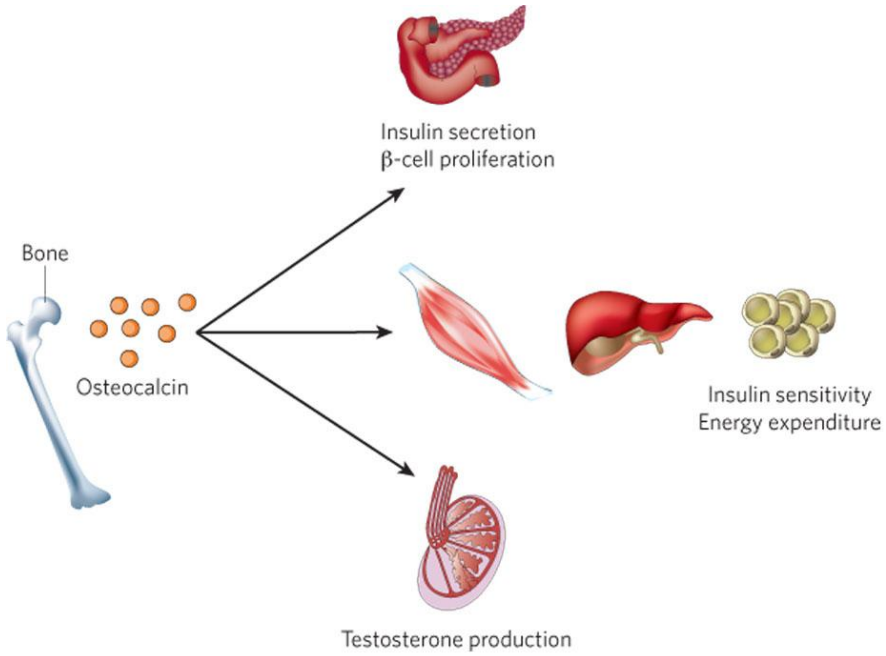


Figure 20.3 *shows the role of osteocalcin in respect of many organs-Image taken from:*
Osteocalcin,a bone -derived multifunctional hormone .
Karsenty G and Ferron M
Nature
2012,481 314-320 .

Methods

The researches carried out relating to both osteocalcin and actin have been performed through online research promoters, the consultation of various texts regarding biochemistry and articles published on the most important scientific journals preserved in various university libraries.
Regarding the initial analysis, performed in order to verify the possible post-translational modifications a protein can undergo following its synthesis, i have consulted several biochemistry textbook. With regards to role of the chaperones for the proper folding of the polypeptides,i have found that this topic is well presented in the textbook "Biochemistry" by Campbell/Farrell (Campbell MK, 2003), where ubiquitination is very well described. The topic of the various degradation system was developed based on the informations provided by the textbook by Zanichelli "The basic of biochemistry"(Champe PC et al. 2006).
Among used texts we can find "Biochemistry" (Campbell MK, 2003) where is evidenced how a protein meets various modifications, before it can reach adequated biological form, to make its proper function. For example, it is put on light how molecular chaperones intervention, are essentials to make an adequate folding of the protein neosynthesis.
In this book are evidenced some methods of protein degradation, as ubiquitination. Another consulted book was the Zanichelli Volume "The basic of biochemistry", (Champe PC et al. 2006) where are evidenced degradation systems of the various proteins determined by specific protease, or for the presence of SUMO proteins , that interfere in episodes of catabolism protein.
Other consulted texts are "Fundamentals of biochemistry" (Voet D et al. 2013) and "Lehningher principles of biochemistry" (Nelson DL et al. 2002),where in both volumes are analyzed the most common post-translational modifications, in which polypeptides occur, after maturation. I found these modifications also in osteocalcin and in the actin.
After examining accurately the post-translational process, that every protein suffer after its synthesis and maturation, through the various texts that i have already mentioned, i studied the biochemical structural analysis of the two proteins taken in exam.
The informations about the primary, secondary and tertiary structure of the proteins were taken from the databases of Swiss-Prot and Uni-Prot, OMIM and Pubmed.
By Swiss-Prot and Uni-Prot i was also able to analyze the various filogenetic aspects of the two proteins. Indeed, by accessing the news reports on these sites, i was able to compare the structural changes of actin occurred since the first forms of life on earth such as fungi, parasites, and mammals such as homo sapiens were detected. Moreover,the structural changes, even the least obvious, that happened in osteocalcin from the simplest and most primitive species as some cephalochordate til highly complex species such as some mammals including humans were founds.
With regards to, the genes the OMIM and GeneCards databases were consulted.
In these sites i have found the genes of the two proteins together with their chromosomal location, together with a clear mapping different according to the various species examined. In addition, by looking at the GeneCards' website it was also possible to learn the starting site of transcription of the six "isoforms" of actin and osteocalcin genes. It was also possible to identify where these genes are mainly expressed in various human tissues. Particularly,on the OMIM website I could get more information on the isolation, characterization and structure of the genes. It was also possible to detect allelic variants of the actin which meet the six "genetic isoforms". Thanks to OMIM it was even possible to learn how the congenital pathologies determined by the advent of these genetic mutations can deteriorate the proper expression of the different "isoforms" of actin.
Finally, for what it concerns the analysis related to the post-translational modifications of actin and osteocalcin correlated to the different life stages in Homo sapiens, gender differences, changes remarkability in different ethnic groups, aspects relating to nutrition and various pathophysiological states. I looked at about a hundred publications on "Pubmed" with respect to the most important journals (Biochemistry,Plos One, Science, EMBO, FASEB journal, Bone, Endocrinology, Nature,

The journal of biological chemistry, Cell, The journal of cellular biology, Development FEBS letters ecc. ecc.)
A few papers, as “Nonenzymatic in corporation of glucose and galactose into brain cytoskeletal proteins in vitro”(Brown MR et al.1992), “Glycation of brain actin in experimental diabetes”(Pekiner C et al. 1993) and “Posttranslational modification of nerve cytoskeletal proteins in experimental diabetes” (McLean WG et al. 1992) were kindly provided by the libraries of other Italian Universities (Bologna, Ancona and Pavia)

Figure 21.1/21.2: *number of publications osteocalcin and actin divided by year.*

Years	*Publications ‰*
1960	-
1961	-
1962	-
1963	-
1964	-
1965	-
1966	-
1967	-
1968	-
1969	-
1970	-
1971	-
1972	-
1973	-
1974	-
1975	-
1976	-
1977	0,004
1978	0,011
1979	0,007
1980	0,036
1981	0,053
1982	0,051
1983	0,069
1984	0,101
1985	0,204
1986	0,227
1987	0,270
1988	0,328
1989	0,472
1990	0,531
1991	0,634
1992	0,630
1993	0,769
1994	0,805
1995	0,811
1996	0,921
1997	0,993
1998	0,964
1999	0,954
2000	0,915
2001	0,903
2002	0,895
2003	1,001
2004	0,938
2005	0,976
2006	0,862
2007	0,832
2008	0,871
2009	0,876
2010	0,929
2011	0,981
2012	0,991
2013	0,940

Years	*Publications ‰*
1960	0.143
1961	0.141
1962	0.159
1963	0.156
1964	0.179
1965	0.176
1966	0.184
1967	0.203
1968	0.202
1969	0.214
1970	0.196
1971	0.412
1972	0.599
1973	0.629
1974	0.614
1975	1.116
1976	1.113
1977	1.211
1978	1.466
1979	1.567
1980	1.857
1981	2.088
1982	2.332
1983	2.477
1984	2.671
1985	2.699
1986	3.213
1987	2.765
1988	2.980
1989	3.461
1990	3.785
1991	4.173
1992	4.252
1993	4.581
1994	4.690
1995	4.859
1996	5.188
1997	5.780
1998	6.030
1999	6.247
2000	6.119
2001	6.085
2002	6.390
2003	6.487
2004	6.434
2005	6.461
2006	6.370
2007	6.305
2008	5.922
2009	5.837
2010	5.725
2011	5.516
2012	5.404
2013	5.202

Figure 21.3: *The graph shows the number of publications from 1960-2013 osteocalcin ‰.*

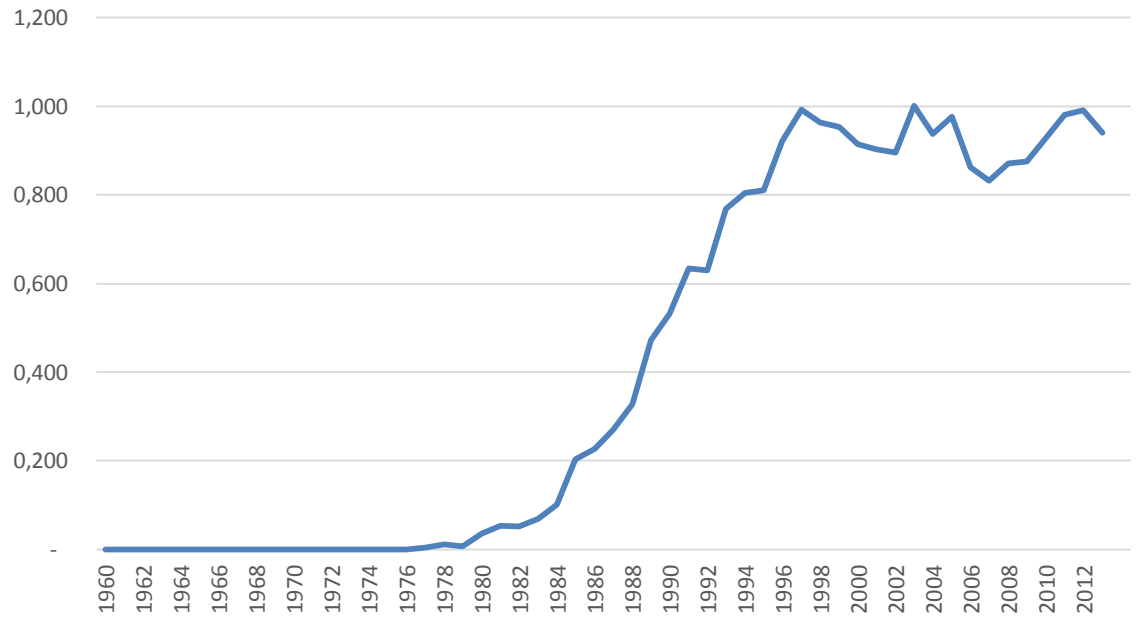
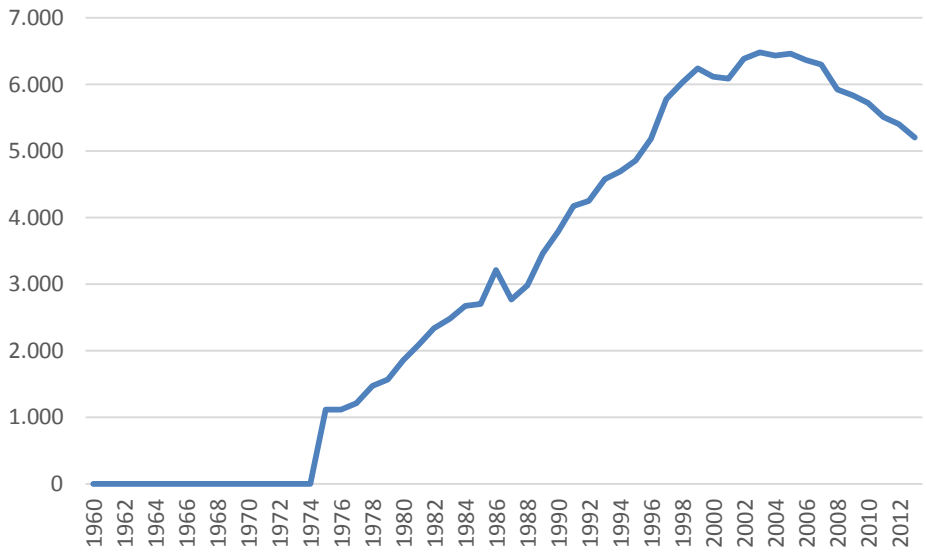


Figure 21.4: *The graph shows the number of publications from 1960-2013 actin ‰.*



Role of expression actin BGLAP

Some of the works carried out during the past 30 years underlined how cytoskeletal proteins and more specifically actin affect the expression of genes involved in bone development like osteocalcin.

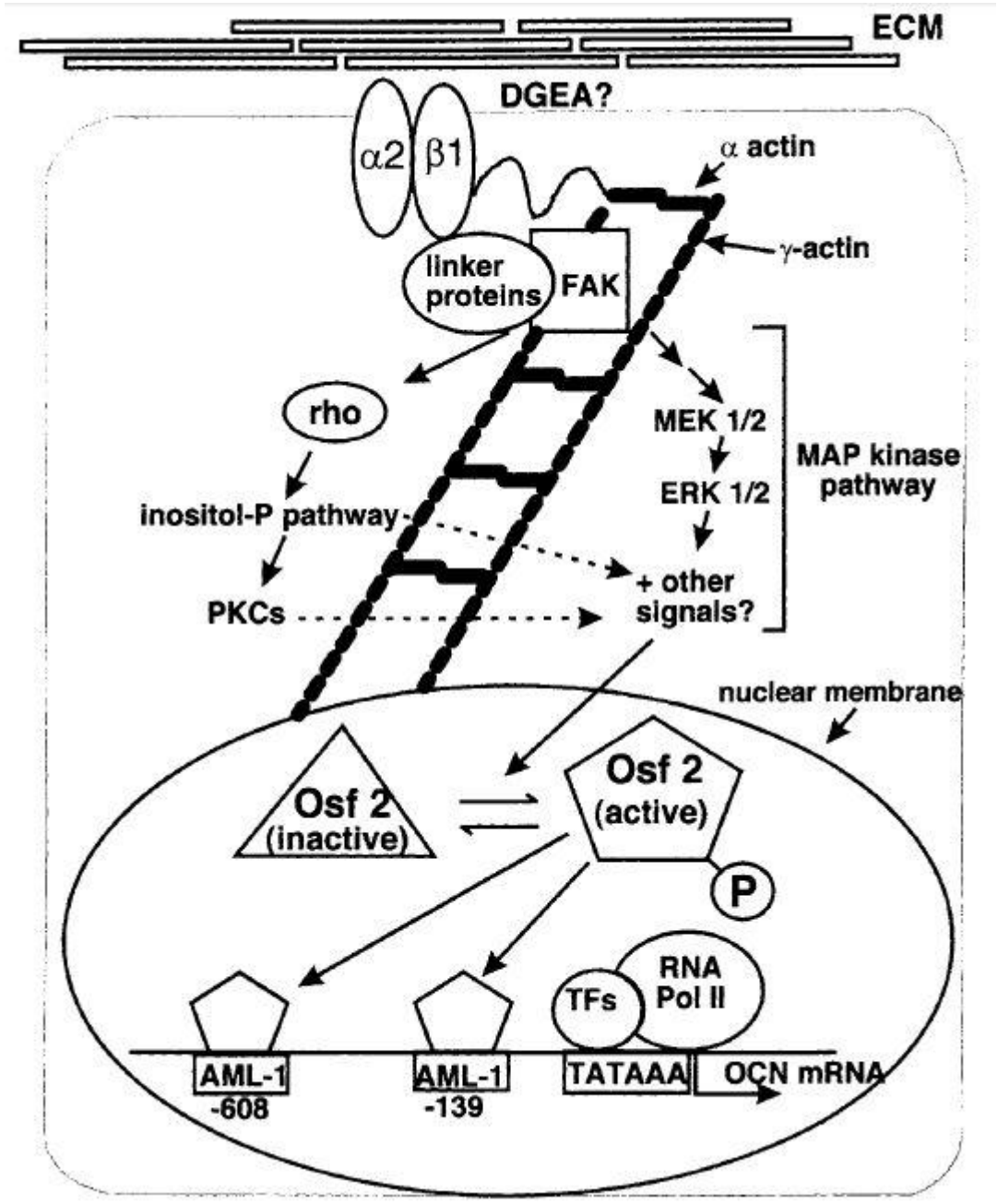
In a study published in 1990, Lomri et al. reported that an increase of the parathyroid hormone (PTH) and Vitamin D in the osteoblastic cells cause considerable changes in the cytoskeleton. (Lomri A et al.1990). They observed that in human bone cells treated with PTH (20 nM per 24 h) the hormone consistently reduces the biosynthesis of vimentin ed α-actin and induces an intracellular increase of cyclical AMP (Lomri A et al.1990). Furthermore, a PTH increase may actually decompose the cytoskeleton bringing a reduction between 53 and 70% of proteins such as actin, α-actinin and α-tubulin (Lomri A et al. 1990). According to this study even a dose of vitamin D of 10 nM in 24 h, contributes to a decline between 40-64% of polymerization of 'α-actinin (Lomri A et al.1990). However, what appears interesting is that these cytoskeleton changes are associated to a rise of 83% in the production of osteocalcin, link to a major expression of the BGLAP gene (Lomri A et al. 1990). In conclusion, this analysis demonstrates how the influence of PTH and vitamin D induce, through bone cells, the inhibition of cytoskeleton proteins like actin and these alterations provoke in turn a functional response in the osteoblastic cells increasing the production of proteins like osteocalcin. (Lomri A et al. 1990).

The role of the extracellular matrix in the differentiation of osteoblasts, was suggested by Franceschi (Franceschi RT 1999). This study highlights how osteoblastic cells monitor the production of ECM (extracellular matrix) to satisfy the ever-changing needs of bone tissues. In particular, the absence of ascorbic acid, a vital element for the hydroxylation of pro-collagen chains and the aggregation of collagen fibrils, hasn't only negative effects on the extracellular matrix but it causes a slighter differentiation of osteoblasts due to the lack of OC gene expression (Togari A et al.1995 and Mahmoodian F et al.1996). Ascorbic acid functions as a cofactor in hydrosilation residues Pro and Lys in the molecules of collagen, promoting the formation of ECM, maturation and collagen deposition. ascorbic acid induces the activity of alkaline phosphatase plasma membrane of osteoprogenitor (Bourne GH, 1948).

In the presence of ascorbic acid, the synthesis of the ECM increases 20 times the expression of OC via the OSE2 (osteoblastic specific binding elements 2) promoter that promotes the binding site for the transcription factor Osf2 / Cbfa1 (osteoblast-specific transcription factors 2/core binding factor alpha 1) (Xiao G et al.1997).

It is interesting to underline that the extracellular matrix communicates with the cell surface via integrins that give rise to an intracellular signaling that induces the expression of genes specific for osteoblasts as BGLAP, in processes such as wound healing, cell migration or morphogenesis (Franceschi RT, 1992). Integrins transmit information, either acting directly on the ECM or with the actin cytoskeleton via actin-binding proteins like talin, vinculin and paxilina that behave as molecules of signal transduction and via tyrosine phosphorylation activating the MAP kinase cascade (Figure 22.1) (Roeckel D et al. 1994 Sudbeck BD et al.1994 and Lin TH et al. 1997). It was shown that the inhibition of integrin function CX2 in MC3T3-E1 cells freezes the promoter for osteocalcin, the activation of Osf2 / Cbfal and in general the differentiation of osteoblasts (Xiao G et al. 1998).

Figure 22.1: *The developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment (Franceschi RT ,1999): In the image is highlighted the interaction between integrins and extracellular matrix and the actin cytoskeleton in order to activate the factors, described previously responsible for the activation of various genes-promoting osteoblast differentiation including BGLAP.*



More recently focus on the changes that occur in osteoblastic cells and the resulting changes in actin in the presence of particular molecules (Boland S et al.1996).

In a first study the role played by isoflavone genistein derived from soy was investigated by analysing the cytoskeleton and the differentiation of bone in ovariectomized rats (Li B et al.2003). It is suggested that genistein causes a change in the level of cytoskeletal F-actin which appears to be essential for endocytosis in osteoclasts, thus leading to an inhibition of osteoclastic bone resorption by promoting the expression of genes responsible for bone differentiation (Li B et al. 2003).

In addition, this work confirms the properties of genistein to protect women in post-menopausal from bone resorption diseases and to promote bone formation (Li B et al. 2003).
A study undertaken in 2009 has further clarified how changes in cytoskeletal dynamics, due to certain factors or substances, can directly affect osteoblast differentiation (Higuchi C et al. 2009). Before this study it was not clear the link between the cytoskeletal organization and gene expression responsible for the osteoblastic cells (Higuchi C et al. 2009).
By using two reagents, known to interfere with the polymerization of actin, cytochalasin D and latranculin B tested on murine cells MC3TE-E1 it was concluded that the expression of genes in osteoblasts is regulated by cytoskeletal reorganization of actin filaments (Higuchi C et al. 2009).
A treatment for a short period with cytochalasin D increases alkaline phosphatase, osteocalcin and secretion of extracellular matrix in the cells MC3TE-E1. The destruction of actin microfilaments induced the phosphorylation of serine residues 744/748 activating PKD(protein kinase D) in a dose-dependent, in turn cytochalasin D suppresses the action of GO-6976 an inhibitor of PKD causing a substantial increase of the expression genes of osteoblastic cells (Higuchi C et al.2009). It was shown that latrinculine B that binds to an actin monomer, preventing actin polymerization, stimulates the expression of proteins such as alkaline phosphatase and osteocalcin (Higuchi C 2009). These data fully confirm as the transient reorganization of the actin cytoskeleton stimulates osteoblast differentiation by increasing the expression of genes such as skeletal protein osteocalcin (Higuchi C et al. 2009).
A further confirmation of how the actin cytoskeleton might affect osteoblast differentiation is reported in 2009 in a study carried out in the UK, where it was demonstrated that the major lipid present in the serum, lisofosfatidic acid (LPA), in conjunction with vitamin D3 stimulates the maturation of human osteoblasts (Mansell JP et al. 2009). Probably, this maturation derives from the fact that osteoblasts undergo a state of peripheral tension due to the reorganization of the actin cytoskeleton by treatment with LPA. However, to confirm this hypothesis Mansell tested on MG63 osteoblast-like cells agents such as colchicine and nocodazole known to act on the cytoskeleton, causing the breakdown of microtubules and polymerization of actin stress fibers. These agents concomitantly with D3 promoted increased osteoblast differentiation (Mansell JP et al. 2009). Also in this case a cytoskeletal remodeling with stress polymerization of actin filaments induces a significant increase of the expression of osteocalcin in MG63 cells (Mansell JP et al. 2009).
As a result of all these observations, it is particularly clear how actin is often responsible for the expression of the osteocalcin

Discussion

The aim of this study was to analyse in two distant molecules in terms of chemical-physical and functional properties such as actin and osteocalcin in order find a possible correlation between the two. Actin is expressed in mammals in six 'isoforms' codified by distinct genes in different chromosomes (Engel JN et al. 1981 and Perrin JB et al.2010) and can be found in its globular (G-actin) or polymerized form to form microfilaments(F-actin)(Greene Le et al. 1984). The protein composed of 374 amino-acids has a mass of 43 kDa and it is found in almost all eukaryotic cells in a concentration higher than 100 µM.
Osteocalcin is expressed in mammals by the BGLAP gene on the chromosome 1 (Puchaz E et al. 1989) and the expression is controlled by Vitamin D (Staal A et al. 1996). The protein is composed of 49 amino-acids for an overall weight of 5.8 kDa. It is present as a non-collagenous protein in bones and dentine (Price PA et al. 1980) it fosters the production of hydroxyapatite, the mineral liable for bone strength (Hauschka P et al. 1989).
My study was conducted by searching the 'Pubmed' website where approximately 100 publications referred to actin and osteocalcin were analyzed. The research was focused on papers regarding post-translational modifications of both molecules. Concerning actin, some of the most relevant modifications were: glycosylation, acetylation, oxidation, ADP-ribosylation and arginylation (Terman JR et al. 2011). However, modifications due to PH variations should not be underestimated (Schonichen A et al. 2013) as well as the cross-link caused by pathogens like Vibrio Cholerae ((Russo L et al. 2013). Moreover post-translation modifications referred to those proteins that bind and interact with actin, the so-called actin-binding proteins, are of great importance. Among the best-known actin-binding proteins there are gelsolin, profilin, ADF-cofilin, DNase I and many others that guarantee a correct working of each body component (Dominguez R et al. 2011). By contrast, for what concerns the modifications occurring in osteocalcin, the most important of these is the γ- carboxylation of glutamic acid (GLA) at residues 17-21-24 catalysed by vitamin K (Hauschka PV et al. 1989), however, the modifications brought by Ca2+to this molecule are equally important (Lee Y et al. 2011), as well as PH variations (Yan W et al. 2013) and the cross-linking that provide the proteolytic cut determining the adequate working of osteocalcin (Schwetz V et al. 2012).
The first point in common between the two proteins, can be summarized as follow: in Homo sapiens both molecules show post-translational modifications vital for the organism growth during all development phases, beginning with the pre-natal stage until reaching senility. Many of these modifications are related to gender differences. Among these, modifications affecting the SABP actin-binding protein are responsible for diseases of the male apparatus (Capkova J et al. 2007) while modifications of the actin-binding protein fascin causes breast cancer in women (Esnakula AK et al. 2014). Concerning osteocalcin, differences in carboxylation are involved in male infertility (Patty A et al. 2013).
A further analogy is the fact that in actin and osteocalcin, post-translation modifications are often in different ethnicities. For instance, it has been noticed that the actin-binding protein fascin is primarily expressed in Afro-American women (Esnakula AK et al. 2014) as we as ACTN4, responsible for a particular renal (Bostrom MA et al. 2012). Differences in carboxylation of osteocalcin (party due to a different nutrition) have been detected in various areas of the world: more carboxylized osteocalcin is present in Asian people compared to Northern Europeans. Another similarity between actin and osteocalcin is that in both cases several post-translational modifications are strictly linked to nutrition (Booth SL et al. 1999, Hamdi HK et al. 2005 and Yu CC et al. 2012) and many can be detected in numerous pathologies. In particular, it has been observed that in both proteins important modifications are present in cardiovascular diseases, diabetes development, renal diseases and infertility.

However, the most important correlation between the two proteins was found in the transcription control since actin is responsible for the monitoring of the osteocalcin gene's expression.
The importance of this function has been confirmed by several studies in the past 30 years reporting how cystoskeletal modifications, of actin and its actin-binding proteins, due to reagents such as cytochalasin D or latrinculin B (Higuchi C et al. 2009) or to isoflavones such as genistein (Boland S et al. 1996), cause different expression of the osteocalcin gene (Lomri A t al. 1990).
Starting from the present study, it can be suggested the possibility to carry out an analogous investigation on other proteins in the future.

Conclusion

With this study, I wanted to compare two intracellular proteins, actin and osteocalcin, different for their chemical-physical properties, functional roles and expression in various tissues. The structural, genetic and pathophysiological aspects of each proteins were accurately investigated, with particular attention to the post-translational modifications. Other informations about possible expression modifications, eventually related to gender, age, ethnicity, and nutritional habits were also taken into account. The study was done by looking for accurate evidences, taken from different databases of public access.

A possible link between actin and osteocalcin came to the light. Indeed, it seems that a number of xenobiotics, such as some isoflavones (i.e. genistein) or other compunds (cytochalasin D and latrinculine B) may cause changes in the cytoskeleton or may interact with actin-binding proteins and be involved in the osteocalcin gene expression.

It is therefore evident that proteins, apparently not related, can in some way find a correlation point, probably relevant to the homeostatic regulation of every living organism. So it is possible to imagine that similar parallel investigations on other molecules apparently not correlated, could bring new light on the mechanistics aspects of biochemistry and physiopathology of life. By this way I believe that this kind of investigations can contribute to enrich our knowledge, in the same way by which the “moonlight proteins” were discovered.

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