Chapter 2

Exploiting Exosomes for Differential Diagnosis of Multiple Myeloma and Monoclonal Gammopathy of Undetermined Significance

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Additional information is available at the end of the chapter

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Abstract

Multiple myeloma (MM) is a plasma cell dyscrasia characterized by a clonal plasma cell proliferation. Usually, all MM are preceded by an asymptomatic premalignant stage termed monoclonal gammopathy of undetermined significance (MGUS). Differential diagnosis requires the evidence of end-organ damage, but recently new biomarkers are emerging to help clinicians to distinguish MM from the premalignant phase. Circulating exosomes in serum seem to be a powerful tool to be analyzed for liquid biopsy, and in this chapter, we show that MM and MGUS exosomes are different in concentration, biological activity, and biochemical markers. These differences seem to be related to the free light chains (FLCs) associated with exosomes and their propathogenic properties. The cellular processing FLC-decorated exosomes and their ability to activate proinflammatory mechanisms are different in MM and MGUS patients. These elements can be evaluated to create an innovative multiparameter panel to monitor MGUS to MM switching.

Keywords: multiple myeloma, exosomes, biomarkers, MGUS

1. Introduction

This chapter aims to show the possibility of differential typization of multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) serum-derived exosomes exploiting an innovative biochemical and bio-physical approach, in order to evaluate new biomarkers for the differential diagnosis among these two conditions.

MM is a plasma cell dyscrasia characterized by a clonal plasma cell proliferation. The worldwide MM incidence is 3.29/100,000 and increases with age. In the 80–84 years old population, the rate is 37.1, while the median age of diagnosis is 73 years [1]. Usually, almost all MM are preceded by an asymptomatic premalignant stage termed MGUS [2]. MGUS is present in roughly 3–4% of the population over the age of 50 years and it is associated with a risk of progression to MM of approximately 1% [3]. MGUS is clinically silent and is usually diagnosed incidentally when a monoclonal (M) protein is detected during laboratory work-up of patients who have a wide spectrum of clinical conditions. The diagnosis of MGUS requires the absence of hypercalcemia, renal failure, anemia, and bone lesions (CRAB features) that can be attributed to the underlying plasma cell disorder.

Thus, over the years, the diagnosis of MM instead required evidence of end-organ damage attributable to the neoplastic clone of plasma cells in order to prevent MGUS patients from unnecessary and toxic chemotherapy. Furthermore, the MM diagnosis requires bone marrow examination like osteo-medullary biopsy to prove the presence of clonal bone marrow plasma cells [3]. Identification of new biomarkers of malignancy is fundamental to prevent end-organ damage in selected patients who are at imminent risk of symptomatic progression.

In this scenario, liquid biopsy could provide an alternative to invasive procedure and improve screening and early detection of cancer [4]. Liquid biopsy is defined as the search of biomarkers [5] in peripheral blood, and in recent years, exosomes have arisen as a powerful tool to understand cancer biology. Exosomes are vesicles with a size of 50–150 nm, which are secreted by cells into the extracellular space and play an important role in cell communication as cargoes of several specific proteins and RNAs. Exosomes are to date considered playing a pivotal role in information transfer in hematological malignancies [6] and recent studies show that specific exosomal microRNAs are involved in pathogenesis and have a prognostic role in MM [7]. In this chapter, we will analyze the biochemical and biological characteristics of MM and MGUS FLC and their cellular processing through exosomes. We show that free light chain (FLC)-decorated exosomes from patients carry peculiar bio-physical and biological qualities that allow the distinction of the malignant and premalignant condition.

2. MM and MGUS FLC cellular processing

In this section, we describe the potential pathogenic properties of serum FLC from MM and MGUS patients, correlating them with exosomal mediated cell-to-cell communication. About 80% of MM originates from intact immunoglobulin, non-IgM, MGUS and 20% from light-chain immunoglobulin MGUS [2]. Since 2009, the FLC quantitative assay (developed from both the Binding Site and Siemens) has been recommended by the International Myeloma Working Group to predict risk of progression in MGUS, the evaluation and management of MM [8]. This immunonephelometric assay allows the quantification of the kappa and lambda light chains untied to the heavy chains in serum [2, 9]. Calculation of the related kappa/lambda FLC ratio is an important parameter because about a third of patients with MGUS and more than 90% of patients with MM have altered FLC ratios that indicate excess production of a clonal FLC by the proliferating plasma cell population [2].

Thus, MGUS and MM patients can show similar serum FLC concentration with comparable FLC kappa/lambda ratio and the presence of FLC in urine is often confirmed by urine immunofixation [10]. Kappa and lambda FLCs have been long-considered a by-product of plasma cells, but evidences show that they possess intrinsic pathogenic characteristics [10] as in the case of AL amyloidosis, light-chain deposition disease, MM, and altered FLC ratio is associated with higher risk of progression in MGUS.

FLCs are able to disrupt the normal physiology of different organs, such as heart, kidney, and lungs. They are occasionally responsible for severe organ damage [11], and these multiple effects are probably related to their primary sequence or posttranslational modification [12] that can alter their biochemical properties, giving them particular affinity for some tissues with respect to others or activating different turnovers inside the cells [10]. This is confirmed for FLC present in serum of patients with MM and MGUS conditions: experiment *in vitro* showed different FLC internalization rate in endothelial, myocardial, and epithelia cell lines for the MM and MGUS FLC. Considering that FLCs circulate in the blood stream and that they interact with the vascular bed to reach the target tissue, Human Vein Endothelial Cells (HVEC), Rat heart myoblast (H9C2), and Human epithelioid cervix carcinoma (HeLa) cell lines are good models for organs usually involved in FLC tissue damage, respectively, for the endothelial, cardiac, and epithelial compartment. In Di Noto et al. [10], cells were incubated with serum of MM and MGUS patients at constant FLC concentration and results showed a faster internalization rate of MM FLC (after 1 h up to 16 h) compared with MGUS FLC in HeLa, HVEC, and H9C2, respectively (**Figure 1**). This suggests that cells are able to uptake FLC from the extracellular environment and FLCs from MM and MGUS patients have different affinity to these cell types even though all of them can be internalized after 16 h of exposure. In contrast, serum from healthy patients, without altered FLC values and kappa/lambda ratio did not show any similar properties probably because it did not contain FLC with propathological characteristics.

Considering these data, we could hypothesize that the interaction of FLC with peripheral districts depends on high-specific receptors expressed on certain cellular lines and/or that each patient has an individual clinical pattern due to the paraprotein molecular structure: thus, the differences between MM and MGUS can be related to peculiar FLC properties that can also alter their processing inside the cells [13].

It has been shown that FLC from AL amyloidosis patients induces oxidative stress and FLC internalization in cardiac fibroblasts enhances sulfatation of secreted glycosaminoglycans (GAGs) suggesting that the intracellular trafficking pathway of FLC could be correlated with the amyloidogenic potential of paraproteins [14]. Differences in FLC from MM and MGUS patients are not limited at the interaction with different cell type, but also their processing inside the cell is diverse: after intracellular internalization, they are rerouted in the extracellular melieu in different form, soluble or via pelletable extracellular vesicles (EVs).

HVEC and H9C2 cells were incubated for 4 h with serum from MM or MGUS patients containing a final FLC concentration of 20 μg/mL. After this initial step, cells were treated with trypsin, an enzyme able to cleave peptide chains, in order to eliminate FLC remained attached at the cell surface. Cells were, then, left in fresh medium (without FLC). After 16 h, the medium was collected

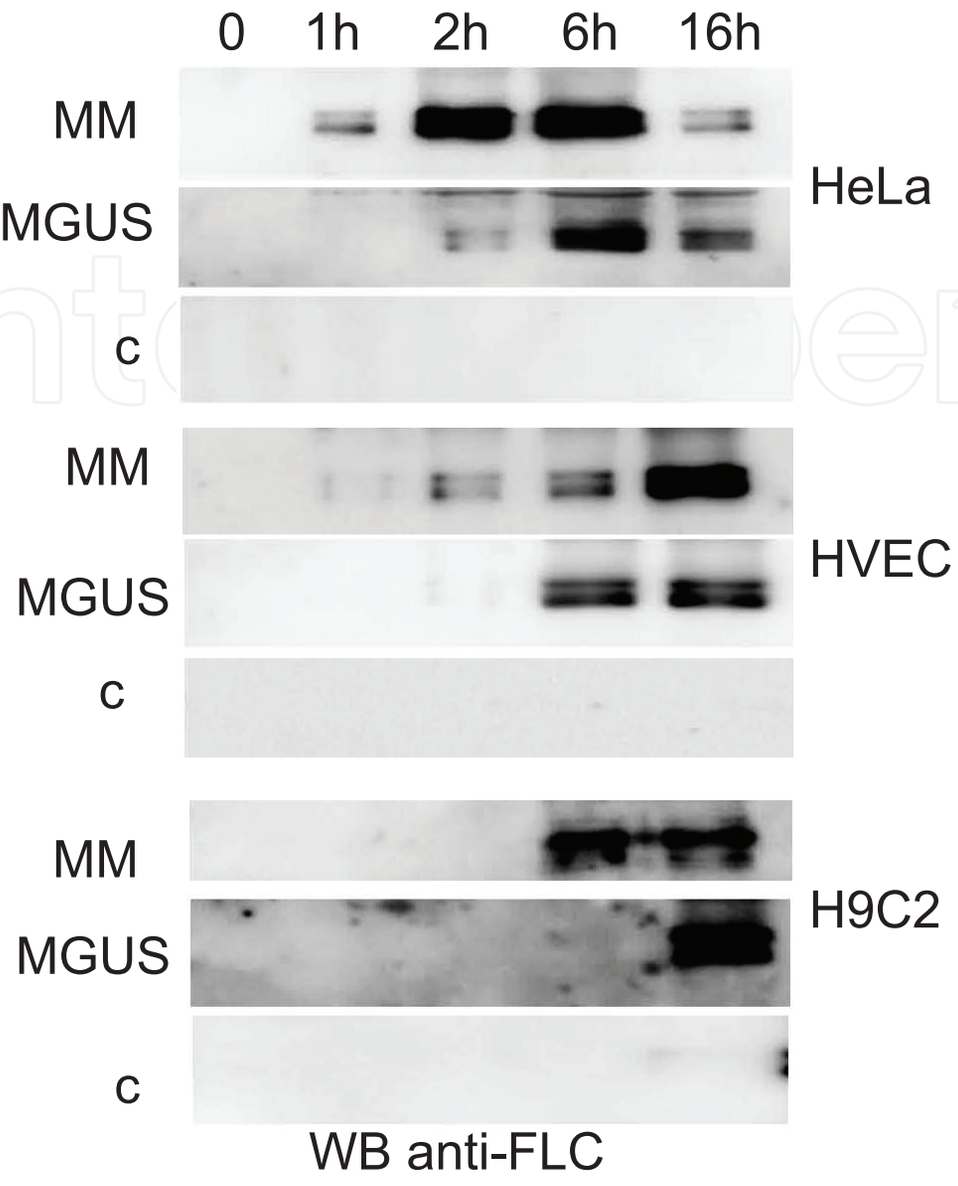

Figure 1. FLCs intracellular uptake. HeLa, HVEC, and H9C2 cell lines were incubated with serum of MM, MGUS at a final FLC concentration of 20 μg/mL and healthy donor (c). After incubation for 0, 1, 2, 6, and 16h cells were processed and analyzed by Western blot with anti-FLC antibody. Adapted from Di Noto et al. [10].

and submitted to a "three step centrifugation" protocol (800 × g 30 min, 16,000 × g 45 min, and 100,000 × g 2 h). This protocol allows the separation of different elements in biological fluids based on their size and density [15], exploiting the gravity force: the first centrifuge allowed to pellet cell debris and large vesicles like apoptotic bodies (P1), the second (P2) microvesicles budded from the plasma membrane (also called ectosomes, with diameters from 150 to 500 nm), while the ultracentrifugation step (P3) pellets smaller extracellular vesicles, like exosomes (50–150 nm) released through multivesicular bodies (MVBs) in the endosomal pathway [15]. FLCs from MM are internalized in cells after 4 h and subsequently released in the medium after 16 h (present in the P3), while MGUS FLCs are only present in cellular homogenate and in the third centrifuge super-

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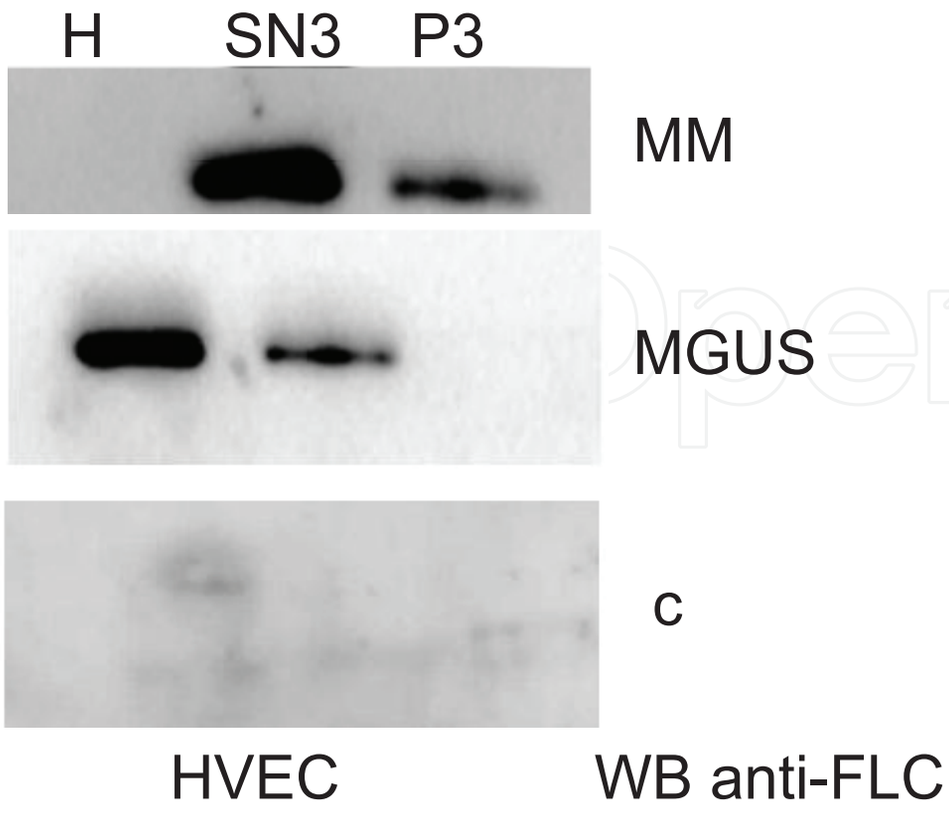

Figure 2. Extracellular rerouting of internalized FLC. HVEC cells were treated as described in the text. H: Homogenate, SN3: supernatant after 100,000 × g centrifugation, P3: pellet after 100,000 × g centrifugation. WB with antibodies anti-FLC. Adapted from Di Noto et al. [10].

natant (SN3). Cell incubated with healthy serum (c) did not give any signal as expected (**Figure 2**). Thus, the MM FLCs, after being internalized, are processed by the cells in a different way than MGUS and released outside the cell in vesicles that are pelleted at 100,000 × g, probably exosomes.

2.1. EVs from cell biochemical characterization

One of the criteria to characterize a population of vesicles and determine their nature is the immunoblot analysis or Western blot (WB) [16, 17]. According to the guidelines, it is important to show that at least three of the common exosomal markers are present in the sample. These protein lists are continuously updating and they include protein involved in exosomes formation (Alix, Hsp70, Annexin V, and Annexin XI), protein enriched in exosomes (tetraspanin CD63, CD81, CD9, and ADAM10) and from the endosomal compartment (TSG101, Syntenin-1, and VPS4B). WB analysis should also include negative controls: signal of protein is not enriched in exosomes (i.e., GM130 for the Cis-Golgi network, Calnexin for the endoplasmic reticulum) to verify the preparation purity from non-endosomal origin membrane. Especially for EVs deriving from cells, it is important to compare on the same gel identical amounts of protein from exosomes and from total lysates prepared from the producing cells to show an enrichment of the markers [16, 18]. In this scenario, Di Noto et al. [10]

showed the presence of the exosomal markers Annexin V, Hsp70, Caveolin 1, Lamp 1, and Tubulin in P3 derived from HVEC and H9C2 culture medium, after MM FLC internalization (**Figure 3**). We observed a Caveolin 1 positive signal in P3 of MGUS treated cells loading four times the amount of MM treated cells. Thus, the P3 preparation from cells contains exosomes and according to the semiquantitative analysis, with different amount from cells exposed to MM or MGUS serum.

It is important to highlight that in this study, Di Noto et al. analyzed the presence of the c-src protein in the cell-derived exosomes. C-src is a tyrosin kinase linked with inflammatory environment and osteolytic bone disease in MM [19] and Di Noto et al. [10] could show that the kinase was present only in exosomes generated during malignant FLC processing, while non-malignant FLCs are unable to induce c-src exosomal recruitment. This is the first

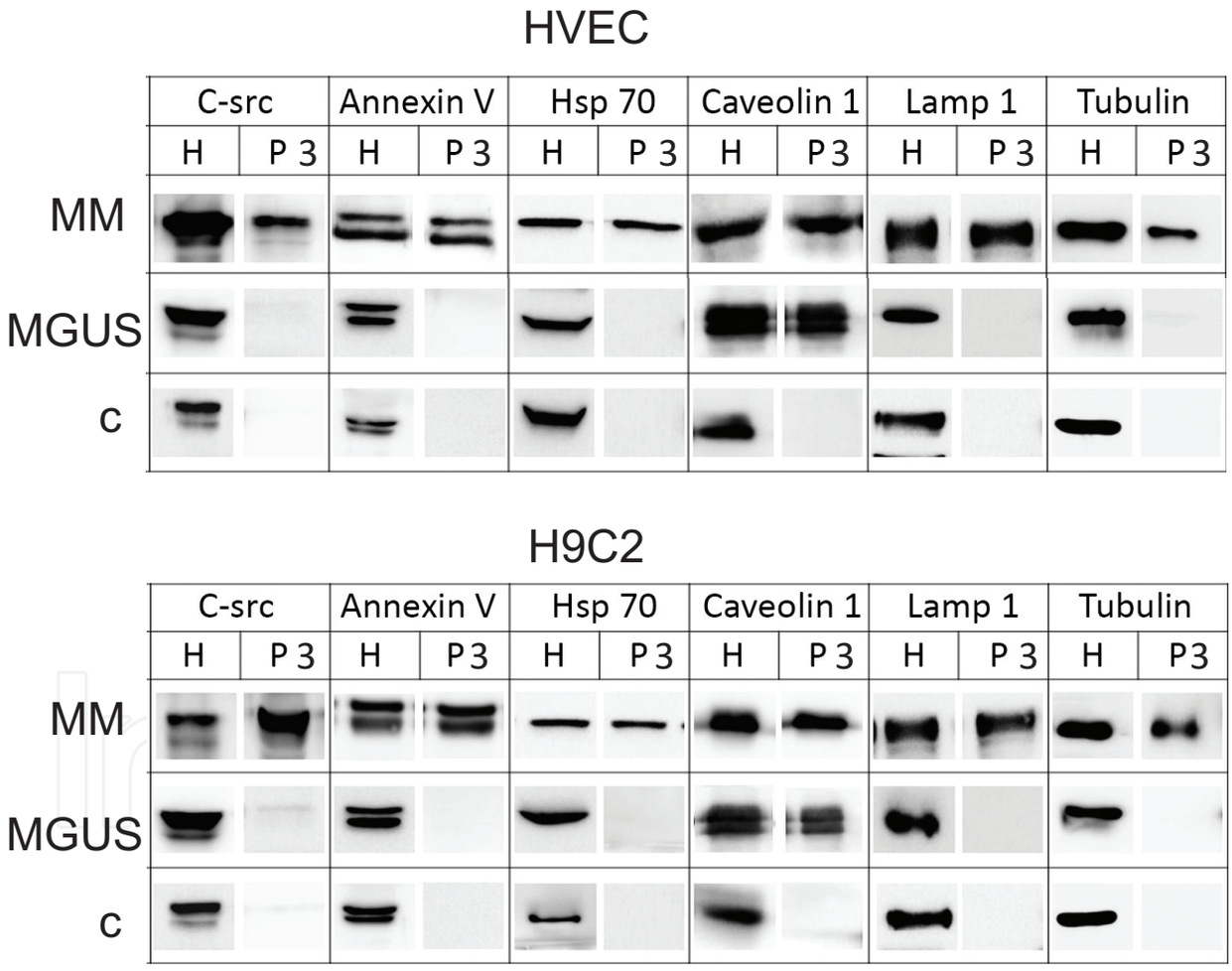

Figure 3. Biochemical characterization of cell-derived exosomes. HVEC and H9C2 cells were incubated with serum from MM, MGUS and healthy donor (c) patients for 4 h at 37°C. The serum was diluted to a final FLC concentration of 20 μg/mL for all samples. Cells were then washed with PBS 1× and treated with trypsin as described in Di Noto et al. and left in fresh medium for 16 h. Medium was harvested, centrifuged at 800 × g for 30 min, 16,000 × g for 45 min and finally, ultracentrifuged at 100,000 × g for 2 h (P3). WB analysis of cell extracts (homogenate, H) and pellets (P3) with different exosome markers (C-src, Annexin V, Hsp 70, Caveolin 1, and Lamp 1). Adapted from Di Noto et al. [10].

evidence of biochemical diversity between exosomes from cells incubated with MM and MGUS serum.

3. EVs detection in MM and MGUS patients' serum

3.1. FLC content of serum-derived EVs

One of the main disadvantages of experiments using immortalized cell lines is that it can be challenging to extrapolate from the results of *in vitro* work back to the biology of the intact organism [20].

For this reason, it was important to confirm the results obtained after studying the FLC processing in human cell lines and verify the presence of the EVs also *in vivo*, analyzing directly patients' serum. After the three step centrifugation protocol on serum, EVs were found in samples from MM and MGUS patients, and MM vesicles show a higher amount of FLC in the P3 fraction than MGUS and healthy patients, estimated around 2% of all the FLC present in serum [10]. These data are consistent with a previous study on urinary exosomes from AL amyloidosis, MM, and MGUS patients [21]. The presence of small amount of FLC in P3 of MGUS serum can be explained with the high variability of EVs production *in vivo* respect of the more restricted type of EVs deriving from a cell culture. Nevertheless, MGUS EVs present different characteristics with respect to MM EVs as explained further in the chapter.

3.2. Serum EVs biochemical characterization

Similar to exosomes from cells, EV preparations from serum must be validated by WB analysis for exosomal markers: P3 from the serum of MM and MGUS patients were blotted for Hsp70, Annexin V, and Tubulin giving positive signals. It is also to note that in the serum, the MGUS exosomes do not contain c-src, confirming that this protein is a marker of malignancy in exosomes from MM patients (**Figure 4A**) [10, 22].

3.2.1. Discontinuous sucrose gradient

Exosomes can be distinct from other type of vesicles for their density, ranging from 1.077 to 1.19 g/mL [6, 16]. Exosomes from MM serum loaded on a discontinuous sucrose gradient from 15 to 60% can be detected in four fractions (from 6 to 9) with density from 1.084 to 1.18 g/mL, according to the markers Hsp70, Annexin V, CD63, TSG101, and CD81 (**Figure 4B**) [6, 10, 22, 23]. FLC and c-src are detected in the same fractions, confirming their association with MM exosomes [10].

In our lab experience and according to guidelines [16], it is best to perform this separation with samples containing high amount of exosomes, for example serum with respect to cellular medium. Usually, exosomes spread in 4–5 gradient fractions and this dilution can decrease

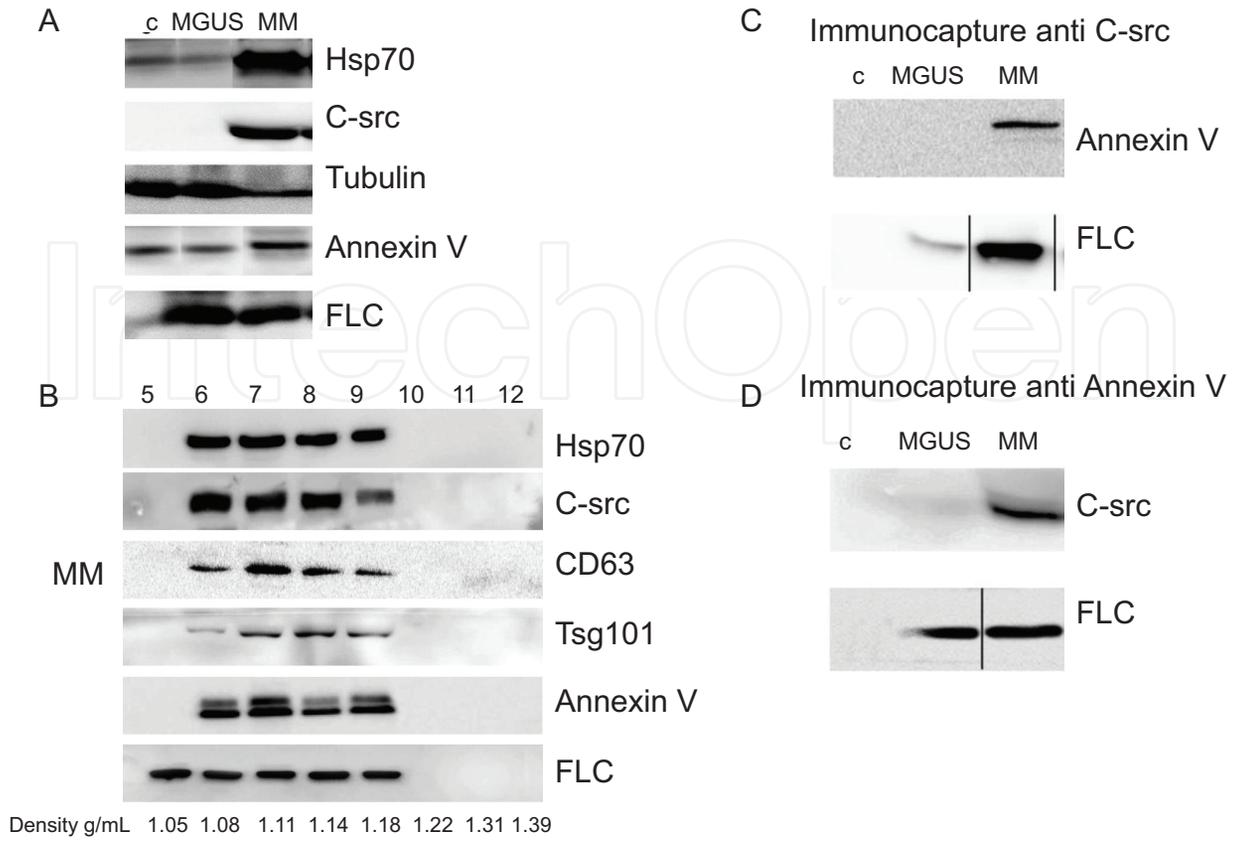

Figure 4. Serum exosome biochemical characterization. (A) P3 obtained from healthy donors (c), MGUS and MM patients were analyzed by WB with different exosome markers. (B) P3 from MM patient was loaded on top of a 15–60% discontinuous sucrose gradient. Twelve fractions of equal volume were collected and analyzed by WB using different exosomal markers and anti-FLC antibodies. Exosomal markers and FLC signals are visible in the same fractions. (C and D) P3 from healthy donors (c), MGUS and MM serum were loaded in a discontinuous sucrose gradient. Fractions from 6 to 9 were collected and incubated with magnetic beads coupled with anti c-src (C) or Annexin V (D) antibodies. Only exosomes deriving from MM serum are both c-src and Annexin V positive. Adapted from Di Noto et al. [10, 22].

the exosomes protein concentration under the limit of detection with available substrate, usually femtomolar, used for the chemiluminescent reaction in WB.

3.2.2. C-src and annexin V MM immunocapture

The immunocapture assay performed with magnetic beads coupled with antibodies anti-c-src (**Figure 4C**) confirmed the ability to precipitate only exosomes (Annexin V positive) from MM serum-containing FLC. On the other hand, anti-Annexin V beads captured MGUS and MM exosomes, but only MM exosomes were positive for c-src signal.

3.2.3. Exosome lipid composition

The lipid composition of the two exosomal populations was analyzed by a thin-layer chromatography showing that both MM and MGUS exosomes are composed by phosphatidylcholine and sphingomyelin, two well-known types of lipids that compose exosome membranes [15]. It is to note that to reveal the P3 MGUS lipids content, we loaded twice the protein concentration than MM P3 (see also Section 3.4).

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3.2.4. Flow cytometry characterization

MM and MGUS exosomes were incubated with FACS magnetic beads coupled to CD63 antibody and stained with a commercial membrane labeler (PKH26). Fluorescent signal was analyzed by flow cytometry revealing the presence of exosomes with the same profile of CD63 expression level [22].

3.3. Serum exosomes morphological analysis

According to the guidelines, in addition to the biochemical characterization described above, to claim the presence of EVs, in particular exosomes, in a preparation, a "single vesicles characterization" is recommended to provide indication of the heterogeneity of the sample [16, 18].

MM and MGUS exosomes preparation both from P3 and gradient fractions were analyzed by atomic force microscopy (AFM), scanning electron microscopy (SEM), and scanning helium ion microscopy (HIM). AFM allows the analysis of vesicles that tend to remain in their original 3D shape. SEM and HIM have a better resolution, but the samples are manipulated. They need to be fixed and dehydrated and these steps can create aggregates and artifacts. In our case, however, all of these techniques allowed to visualize vesicles ranging from 50 to 300 nm according to the heterogeneity of the sample: P3 contains exosomes and bigger vesicles, while gradient fractions are more monodispersed with a size range between 50 and 150 nm (**Figure 5**).

3.4. MM and MGUS exosome quantification

Exosome quantification in a sample is one of the most discussed issues among experts in the field. How is it possible to quantify accurately something with the heterogeneous composition (lipids, protein, and nucleic acids) among a mixture of similar elements (protein aggregated from serum, circulating RNA, and vesicles with similar size, but different origin)?

Bradford assay was used to determine the amount of the total exosomes protein in the samples, and with this method, P3 from MM serum had almost double protein concentration than P3 from MGUS and healthy patients.

Further on, it has been discovered that exosome preparation protein content can be influenced by many elements, i.e. fetal bovine serum in culture medium, single, and aggregated proteins. One of the best solutions is to purify the sample as much as possible from contaminants before every type of measurements, i.e. with a sucrose gradient. Even though what is Bradford assay measuring: protein outside or inside exosomes, or both?

Thus, we decide to measure the activity of an enzyme known to be enriched within exosomes, acetylcholinesterase [24], in a P3 preparation from healthy, MGUS, and MM serum. This assay confirmed that MM P3 preparation contains more exosomes than MGUS and controls, but how much is it specific?

Our lab contributed to solve this set of problems with a nanotechnological approach. We developed a cost-effective and fast colorimetric assay for probing protein contaminants and determining the concentration of EV preparations [25].

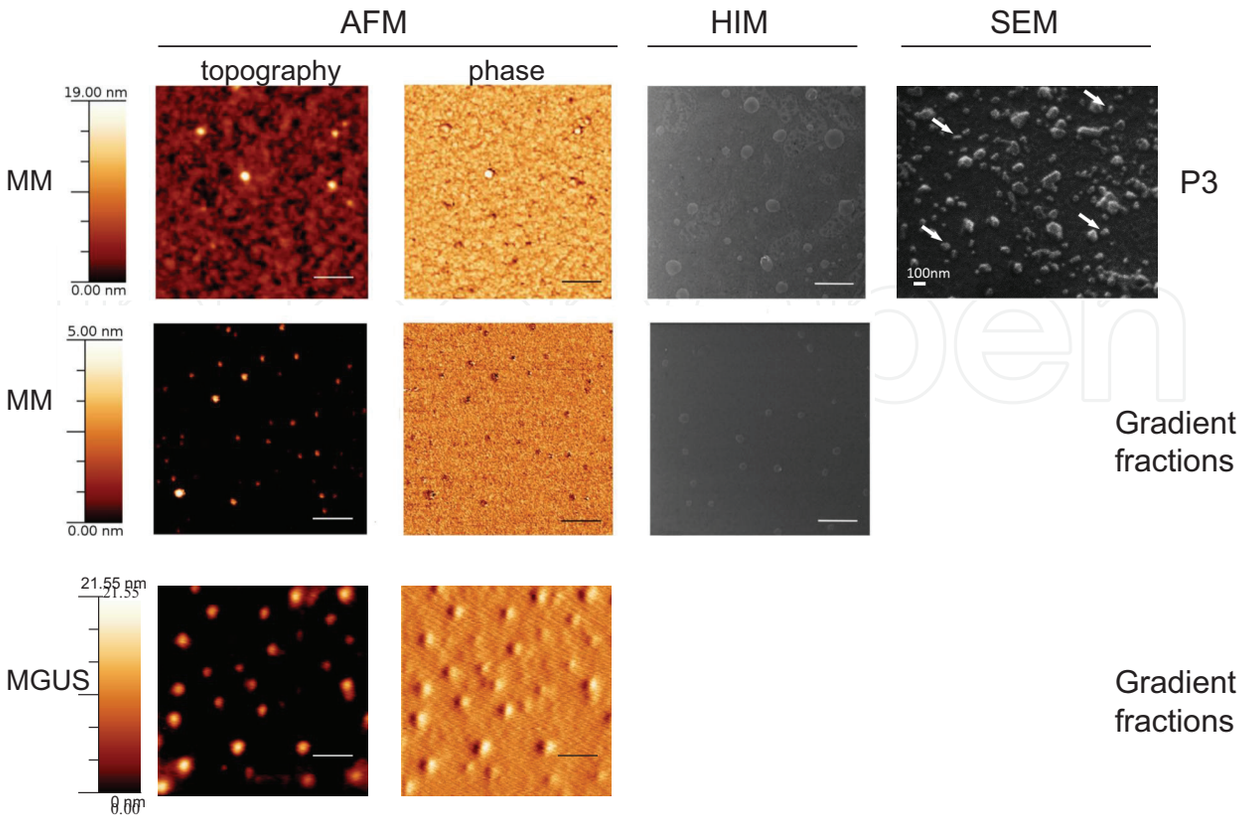

Figure 5. Serum exosome morphological analysis. P3 from MM serum were analyzed by AFM, HIM, and SEM in order to visualize vesicle populations. MM and MGUS sucrose gradient fractions from 6 to 9 were examined by AFM (topography and phase mode) and HIM (only MM sample). MM scale bars are 300 nm for AFM pictures, 500 nm for HIM pictures, and 100 nm for SEM picture. MGUS scale bars are 700 nm for AFM pictures. Adapted from Paolini et al. [6] and Di Noto et al. [10].

The assay exploits colloidal gold nanoplasmonics and the fact that nanoparticle (NP) aggregation at lipid membranes is modulated by the presence of a protein corona around the NPs. When a pure exosome preparation is incubated with a gold NP solution, the NPs cluster at the exosome membrane. Clustering is associated with NP LSPR red-shift, which is proportional to the exosome molar concentration and using a calibration line made of phosphatidylcholine liposome, can therefore be exploited for titrating the solution. If the preparation is not pure from single or aggregated proteins, NPs tend to interact with these elements and do not aggregate on exosome surface. Thus, to obtain a precise exosome quantification, as described, sample must be as pure as possible.

This assay, moreover, can determine the sample purity from protein contaminants with a limit of detection of 0.005 μg/μL.

Using this assay, we probed MM and MGUS exosomes collected from the sucrose gradient fractions from 6 to 9, and we could state that exosome preparations were pure from protein contaminants. In this way, our assay could titrate the exosome concentration in each sample and results showed a four-fold increase of exosomes in MM preparation in comparison with MGUS and healthy control (**Figure 6**) [23]. These data confirmed the previous analyses of exosomes from serum and it corresponds to the production of exosomes in the cells after MM and MGUS serum exposure.

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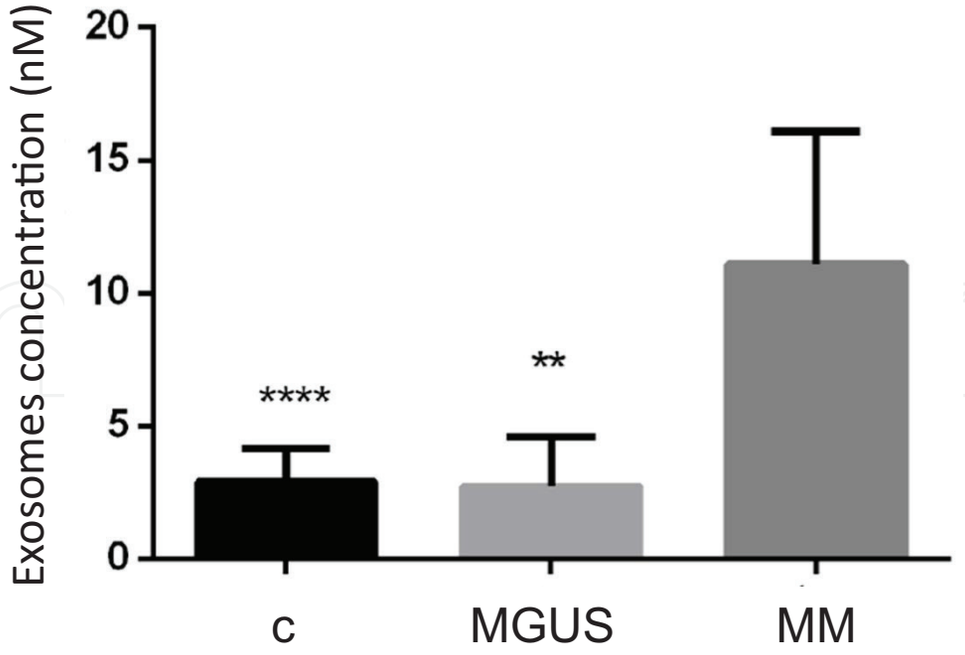

Figure 6. MM and MGUS exosome serum quantification. Exosomes were titrated using the colorimetric nanoplasmonic assay as described in Maiolo et al. [25]. Each column represents the mean value of exosomes isolated from serum of different patients (10 healthy donors (c), 5 MGUS and 10 MM) +/− Standard deviation. Student's t-test p value (p): **** p < 0,0001, *** p < 0.01. Adapted from Di Noto et al. [23].

4. MM and MGUS exosome biological effects

Exosome role in MM is started to be unveiled in the past few years. MM-derived EVs have been demonstrated to have a biological effect on other cell types, such as to induce phenotypical changes in osteoclasts, influence bone marrow microenvironment, and promote the production of proangiogenic factors, like IL-6 and VEFG, in endothelial cells [26].

Interestingly, MM and MGUS serum-derived exosomes showed different biological effects on endothelial and myocardial cell lines: MM exosomes have a higher internalization rate than MGUS and treatment with MM exosomes was able to induce a significantly higher proliferation rate compared with MGUS in both cell lines.

This effect is dependent on active exosomes endocytosis in cells, mediated by the presence of specific pathogenic FLC on the surface of MM exosome as demonstrated with immunofluorescence and cytofluorometry analyses (**Figure 7A and B**). In fact, MM exosomes incubated with antibodies anti-FLC, which mask the interaction with cells, decrease their internalization rate and the induced cell proliferation in both cell lines (**Figure 7C**). Furthermore, MM exosomes docking and processing by cells is influenced by the presence of heparan sulfate proteoglycans (HSPGs) on the cell surface. Incubating MM exosome with heparin, a structural analog of HSPGs, saccharide chains, decreased MM exosome uptake in HVEC cells. MM exosome/heparin interaction is being confirmed in dose-response experiments at surface plasmon resonance (SPR) spectroscopy. These data revealed a probably cooperative binding mechanism of FLC and HSPGs even if exosomes, which are characterized by different FLC decorations, have distinct binding affinities for cell-associated HSPGs and this is reflected in different cellular uptake [22, 23].

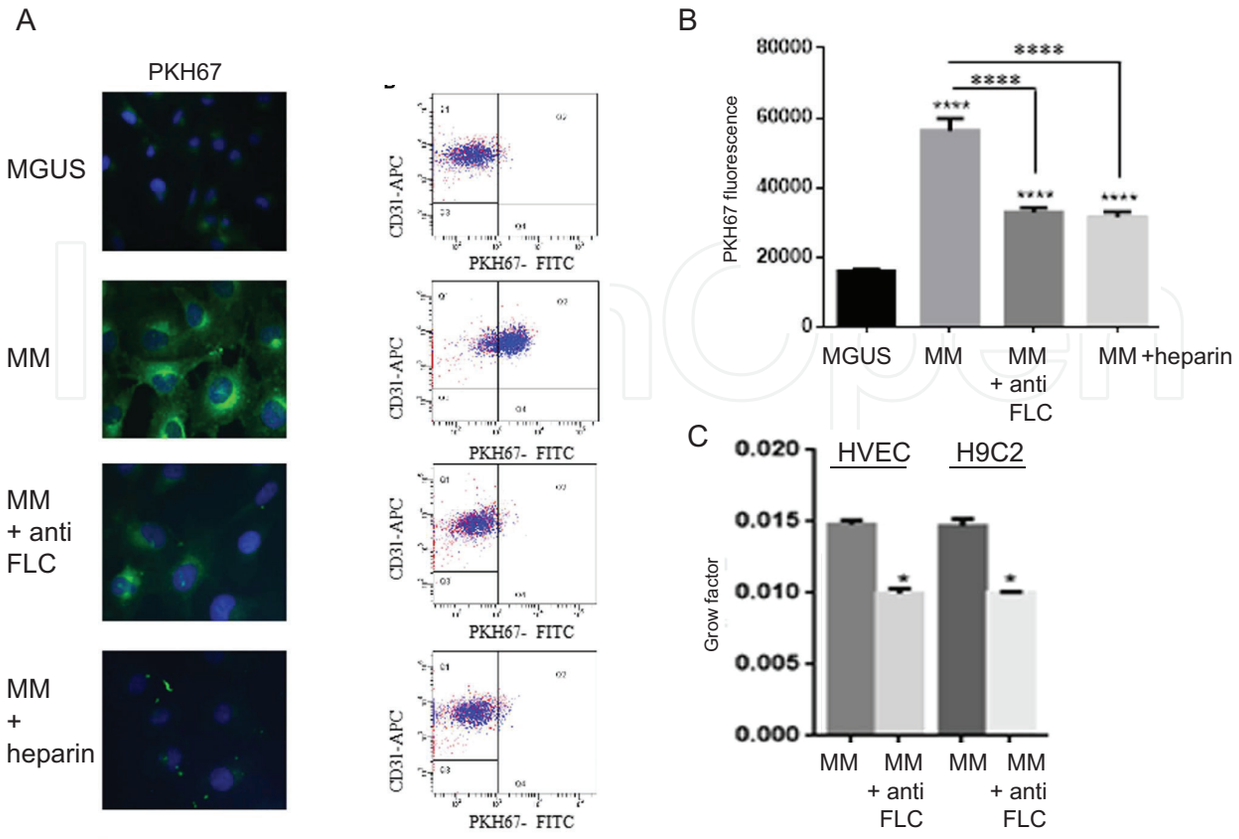

Figure 7. MM exosome biological effect is FLC and HSPG mediated. (A) Fluorescent microscopy and flow cytometry analyses of HVEC cells incubated with MGUS or MM PKH67-labeled exosomes. To demonstrate the FLC and HSPGs involvement in MM exosome uptake, MM vesicles were incubated with anti-FLC antibody (MM+ anti FLC) or heparin before cells incubation. Scale bars 5 μm. (B) PKH67 fluorescence intensity measurement of internalized exosomes from MGUS and MM serum. MM exosomes uptake in HVEC cells decrease after anti-FLC antibody (MM+ anti FLC) or heparin treatments. (C) Proliferation induction by MM exosomes is decreased after incubation with anti-FLC antibody both in HVEC and H9C2 cell lines. Adapted from Di Noto et al. [22].

It has been demonstrated that MM EVs are related to proinflammatory environment in cells. Immunofluorescence and biochemical separation techniques demonstrated that cells treated with exosomes from MM serum show a c-src intracellular redistribution at the plasma membrane with respect to MGUS exosome treatment. These data are consistent with cellular *ex novo* secretion of c-src positive exosomes after MM serum exposure and this phenomenon is reduced after cellular uptake inhibition, incubating MM exosomes with anti-FLC antibodies and heparin.

Similarly, the activation and nuclear translocation of the transcription factor Nf-kB, involved in the cellular responses after stress, infection, and inflammatory stimuli, is not visible in the cell treated with MGUS exosomes. MM exosome's induced effect is decreased after treatment with anti-FLC antibody and heparin (**Figure 8**).

It is to note that the Nf-kB translocation is dependent on the preparation purity: the biological activity of exosome preparations from MM patient serum is influenced by residual contaminants, which may escape the purification procedure. These contaminants probably can interfere with the exosomes-cell membrane interaction or inhibit the Nf-kB translocation. They can be

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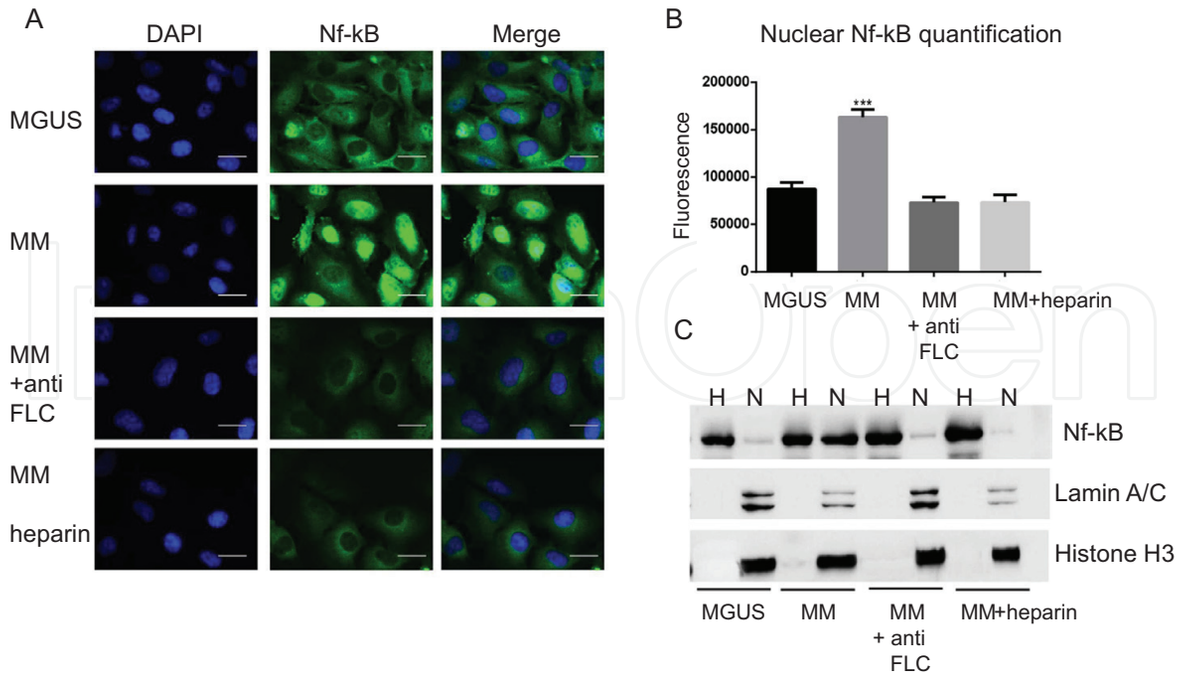

Figure 8. Nf-kB nuclear translocation is MM exosomes induced. (A) HVEC immunofluorescence imaging of Nf-kB nuclear translocation after incubation with MGUS, MM exosomes, or MM exosomes incubated with anti-FLC antibody (MM + anti FLC) or heparin (MM + heparin). Cell were fixed and incubated with DAPI for nuclear staining and anti-Nf-kB antibody as described in Di Noto et al. [22]. Scale bars 5 μm. (B) Quantification of nuclear Nf-kB fluorescence intensity in HVEC cells after MGUS, MM exosomes, MM exosomes incubated with anti-FLC antibody (MM + anti FLC) or with heparin (MM + heparin) treatment. (C) WB analysis of cell extracts (H) and nuclear compartment (N) was performed with anti Nf-kB antibody and nuclear markers (Lamin A/C, Histone H3) after different incubations. Adapted from Di Noto et al. [22].

separated from exosomes after a discontinuous gradient, but they cannot be detected with conventional techniques (i.e. WB) and need a combination of proper bioanalytical methods and nanoscale characterization [6].

5. Conclusion

In this chapter, we have shown that MM and MGUS patients can be distinguished at the nanoscale level. Although their FLC serum content may be similar, FLC biochemical characteristics are very different: they are internalized and processed in cells in distinct ways and only pathogenic FLCs are rerouted in the extracellular compartment in c-src positive exosomes. It is important to note that exosome presenting MM FLC activates a proinflammatory process: the presence of circulating c-src positive exosomes has been demonstrated only in serum of MM patients, electing this kinase as a new marker of malignancy for the differential diagnosis between MGUS and MM. Similarly only MM exosomes have the ability to induce Nf-kB translocation in cells. These parameters could be implemented with the exosome concentration in serum, binding affinity with heparin and ability to induce proliferation in HVEC and H9C2 cell lines to create an innovative multiparameter panels to monitor MGUS to MM switching. We are aware that these data need to be confirmed in a larger cohort of patients and

we still do not know how precocious these phenomena are with respect to the MGUS to MM switch using of the actual diagnostic criteria. Recently, new biomarkers are emerging to help clinicians to distinguish MM from the premalignant phase. According to recent guidelines, extreme bone marrow clonal plasmacytosis (>60%), marked elevation of serum FLC ratio of 100 or higher (provided involved free light-chain level ≥100 mg/L) and/or presence of more than one focal lesion on whole-body magnetic resonance imaging (MRI) can defined as MM even in the absence of CRAB features [2, 3].

In the future, monitoring MGUS and MM exosome parameters could support, as a liquid biopsy, the classical classification methods and help in early diagnosis to prevent the development of end-organ damage for patients who are at the highest risk [2, 3].

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