Heat Shock Protein 60 Levels in Tissue and Circulating Exosomes in Human Large Bowel Cancer Before and After Ablative Surgery

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BACKGROUND: Heat shock protein 60 (hsp60) is a chaperonin involved in tumorigenesis, but its participation in tumor development and progression is not well understood and its value as a tumor biomarker has not been fully elucidated. In the current study, the authors presented evidence supporting the theory that Hsp60 has potential as a biomarker as well as a therapeutic target in patients with large bowel cancer. METHODS: The authors studied a population of 97 subjects, including patients and controls. Immunohistochemistry, Western blot analysis, and quantitative real-time polymerase chain reaction were performed on tissue specimens. Exosomes were isolated from blood and characterized by electron microscopy, biochemical tests, and Western blot analysis. RESULTS: Hsp60 was found to be increased in cancerous tissue, in which it was localized in the tumor cell plasma membrane, and in the interstitium associated with cells of the immune system, in which it was associated with exosomes liberated by tumor cells and, as such, circulated in the blood. An interesting finding was that these parameters returned to normal shortly after tumor removal. CONCLUSIONS: The data from the current study suggested that Hsp60 is a good candidate for theranostics applied to patients with large bowel carcinoma and encourage similar research among patients with other tumors in which Hsp60 has been implicated. Cancer 2015;121:3230-39. © 2015 American Cancer Society.

KEYWORDS: heat shock protein 60 (Hsp60), colon adenocarcinoma, exosomes, plasma cell membrane, natural killer cells, macrophages, theranostics.

INTRODUCTION
Heat shock proteins (Hsps) can be active players in carcinogenesis as well as promising targets for anticancer therapy. Among Hsps involved in tumorigenesis, the 60-kilodalton chaperonin (Hsp60 or HSPD1) is intriguing and is currently considered to be of special interest because its levels are increased in various cancers but are normal or decreased in others. Moreover, it has been shown that it can stimulate or inhibit tumor cell apoptosis, depending on the mechanisms of its accumulation in the cytosol.

Colorectal adenocarcinoma is one of the most studied tumors worldwide. Consequently, clinicians currently possess information regarding some of the molecular mechanisms that trigger the onset of colon cancer and are able to apply better criteria than in the past for prevention and early diagnosis. Nonetheless, a large number of cases are diagnosed every year and patients have to undergo surgical intervention for tumor removal as well as postsurgical therapy, which requires careful follow-up.

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Theranostics is a modern field of medicine that provides a useful platform for physicians to examine molecular markers that might also be potential therapeutic targets or agents, and therefore can be useful for both diagnosis (including early diagnosis and the detection of disease recurrence during follow-up) and therapy. In this regard, exosomes are considered to be promising biomarkers for tumor theranostics for various reasons: 1) their content (including proteins, nucleic acids, and lipids) is a type of fingerprint of the cell from which they originate, and of its status; 2) they can circulate throughout the body; and 3) they have various effects on target cells. 12-15

In the past, we demonstrated in vitro that Hsp60 is present in exosomes released by tumor cells but not in exosomes released by their normal counterparts.16 Moreover, we demonstrated that Hsp60 secretion by tumor cells is a multistage process that includes its: 1) accumulation in the cytosol; 2) translocation to the plasma membrane; 3) internalization by lipid rafts into multivesicular bodies; and 4) secretion via exosomes, in which they localize at the membrane level so it is a potential ligand for numerous types of receptors. 17

Receptors for Hsp60 have been described on various inflammatory cells. The most studied receptors for Hsp60 are toll-like receptor (TLR) 2 and TLR-4 and CD14, CD30, and CD54, which are present mainly on lymphocytes and macrophages. 18,19 It was demonstrated in vitro that exosomes derived from resistant tumor cells treated with anticancer drugs confer immunogenicity by inducing Hsp-specific natural killer (NK) cell responses. 20 In particular, it was found that Hsp-bearing exosomes are able to elicit effective NK cell antitumor responses, which open new avenues for finding a novel strategy for cancer immunotherapy.

In the current study, we investigated in vivo Hsp60 expression, levels, and localization in colorectal adenocarcinomas in comparison with their normal tissue counterparts. We focused on the levels of Hsp60 in exosomes obtained from the blood of patients before and after surgery. These results support the theory that Hsp60 can be considered a candidate theranostic tool for colorectal cancer.

### MATERIALS AND METHODS

#### Patient Recruitment

Subjects were recruited at the Department of Oncological Surgery at the University Hospital of Palermo from April 2011 to March 2014. The local ethics committee approved the study and written informed consent was obtained from each subject. We recruited 57 subjects (26 women and 31 men) diagnosed with colorectal adenocarcinoma who were considered eligible for surgery. Clinical characteristics of these patients are summarized in Table 1. Detailed clinical data are presented in Supporting Table 1. None of the patients, including those with rectal cancer, received neoadjuvant therapy before surgery.

Two blood samples were obtained from each subject, one on the day of the surgery and a second on the day (usually 1 week after) of release from the hospital. These samples were used for exosome isolation. A sample of the tumor mass was taken from the surgical specimen from the same subjects. For comparison, we recruited 40 age-matched subjects (18 women and 22 men) who underwent colorectal endoscopy for screening but did not demonstrate any signs of tumor, inflammatory diseases, or polyps. We obtained a small fragment of colon mucosa and a blood sample from each of these subjects for use as the control group.

#### Tissue Processing

Tumor and normal colon tissues were in part used for messenger RNA (mRNA) and protein extraction and in part for immunomorphological analyses. Tissues for

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**TABLE 1. Summary of Clinical Characteristics of the Patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (54.4)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (45.6)</td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>3 (5.2)</td>
</tr>
<tr>
<td>50-59</td>
<td>7 (12.3)</td>
</tr>
<tr>
<td>60-69</td>
<td>18 (31.6)</td>
</tr>
<tr>
<td>70-79</td>
<td>21 (36.8)</td>
</tr>
<tr>
<td>&gt;79</td>
<td>8 (14.3)</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
</tr>
<tr>
<td>Colon, right</td>
<td>18 (31.6)</td>
</tr>
<tr>
<td>Colon, left</td>
<td>27 (47.4)</td>
</tr>
<tr>
<td>Rectum</td>
<td>12 (21.1)</td>
</tr>
<tr>
<td>pT classification</td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>19 (33.3)</td>
</tr>
<tr>
<td>T3-T4</td>
<td>38 (66.7)</td>
</tr>
<tr>
<td>pN classification</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>20 (35.1)</td>
</tr>
<tr>
<td>N1-N2</td>
<td>57 (64.9)</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>20 (35.0)</td>
</tr>
<tr>
<td>III</td>
<td>37 (65.0)</td>
</tr>
<tr>
<td>R classification</td>
<td></td>
</tr>
<tr>
<td>R0</td>
<td>57 (100)</td>
</tr>
<tr>
<td>R1-R2</td>
<td>0</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td>1 and 2</td>
<td>38 (66.6)</td>
</tr>
<tr>
<td>3</td>
<td>19 (33.3)</td>
</tr>
</tbody>
</table>

*See Supporting Information Table 1 for more detail.

UICC stage system.

UICC grade system.
molecular analyses were frozen with liquid nitrogen and stored at -80°C until use. Tissues for immunomorphology were fixed in formalin and paraffin in 5-μm sections for use in immunostaining. Finally, a small fragment of fresh tissue was fixed in 4% paraformaldehyde and 0.5% glutaraldehyde Karnovsky solution (prepared in 0.1 M sodium cacodylate buffer [pH 7.4]) and embedded in epoxy resin for transmission electron microscopy (TEM), as described below.\textsuperscript{21}

### Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described.\textsuperscript{22} Briefly, total RNA was isolated from 500 mg of tumor and normal colon tissues using TRI Reagent (Sigma-Aldrich, Milan, Italy) according to the manufacturer's instructions. Approximately 300 ng of RNA was retrotranscribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif.). qRT-PCR analyses were performed using GoTaq qPCR Master Mix (Promega Corporation). The mRNA levels were normalized to the levels obtained for hypoxanthine phosphoribosyltransferase 1 (HPRT1), beta-glucuronidase (GUSB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The cDNA was amplified using the primers indicated in Table 2, and the Rotor-Gene 6000 Real-Time PCR Machine (Qiagen GmbH, Hilden, Germany). Changes in the transcript level were calculated using the 2\(^{-ΔΔCt}\) method as described by Schmittgen and Livak.\textsuperscript{23}

### Primary Antibodies

The primary antibodies used for immunoblotting and immunomorphology determinations are listed in Table 3.
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Among 20 patients and 20 controls selected at random, DIF was performed on 5-μm paraffin sections using the following secondary antibodies: antimouse immunoglobulin (Ig) G conjugated with fluorescein isothiocyanate and diluted at 1:200 (Sigma-Aldrich, St. Louis, Mo); antimouse IgM (m-chain-specific) conjugated with fluorescein isothiocyanate and diluted at 1:100 (Sigma-Aldrich); and antirabbit IgG conjugated with tetramethylrhodamine and diluted at 1:200 (Genetex Inc, Irvine, Calif). Appropriate positive and negative controls were run simultaneously. For Hsp60 IHC, sections of normal human kidney were used as a positive control (data not shown). IEM was performed in 10 patients and 10 controls selected at random using the following secondary antibody: 10 nm of gold-labeled goat antimouse IgG (Amersham Pharmacia, GE Healthcare Life Science) at a dilution of 1:30.

IHC results were evaluated by 2 independent expert observers (F.C. and F.R.) who examined the specimens in a blind (code-marked) approach and performed a quantitative analysis to determine the percentage of cells positive for Hsp60. All the observations were made at a magnification of ×400 and the means of duplicate counts were used for statistical analyses.

Exosome Isolation From Blood
Exosome isolation from blood was obtained by differential ultracentrifugation, as described by Caby et al. Ten mL of whole blood was collected from each patient and control subject and plasma was separated by centrifugation at 1800 × g for 30 minutes at 22°C. The plasma obtained was diluted with a phosphate-buffered saline solution and differentially centrifuged at 29,500 × g for 20 minutes at 4°C to eliminate cellular debris. The resulting supernatant fluid was filtered through a 0.2-μm filter. The filtrate was then ultracentrifuged at 110,000 × g for 2 hours at 4°C. The resulting exosomal pellet was resuspended in a phosphate-buffered saline solution or in a radioligand assay buffer solution and stored at -80°C until use.

Exosomal Fraction Assessment
The assessment of the exosomal fraction was performed by TEM, acetylcholinesterase (AChE) assay, and WB analysis, as described.

Enzyme-Linked Immunosorbent Assay Tests
Enzyme-linked immunosorbent assay (ELISA) was performed as described in all patients and controls. We used a commercial human Hsp60 ELISA kit (Enzo Life Sciences, Inc, Farmingdale, NY) and absorbance was measured at 450 nm in a microplate photometric reader (GDV, Milan, Italy).

Carbonate Test
To determine whether Hsp60 was associated with the exosomal membrane, the carbonate test was performed using published techniques, and modified as previously described. Hsp60 was revealed by WB analysis performed before and after carbonate treatments.

Statistical Analyses
Data analyses were performed using the StatView SE Graphics program (Abacus Concepts Inc, Berkeley, Calif). Comparisons between groups were performed using the Student t test for unpaired data. A P value ≤.05 was considered to be statistically significant. Analysis of variance tests for unbalanced data were also performed to verify whether there was a statistical correlation between Hsp60 IHC levels and clinical variables (pT classification, pN classification, and tumor grade).

RESULTS

Hsp60 Expression and Levels in Tumor and Healthy Tissue Samples
Both Hsp60 expression (mRNA as evaluated by qRT-PCR) and levels (protein as evaluated by WB analysis) were found to be increased in tumor samples compared with controls (Figs. 1A and 1B). These differences were statistically significant. The increase in the Hsp60 protein levels was higher than expected from the observed increase in the corresponding mRNA, which is in keeping with the known lack of correlation between these 2 parameters in many other systems, a discrepancy that is believed to be caused by various factors.

IHC demonstrated increased levels of Hsp60 in tumor samples compared with controls. A strong diffuse cytoplasmic positivity for the Hsp60 protein was present in 100% of the tumor specimens examined, with a mean positivity noted among 91.2% of the tumor cells, whereas in normal controls, Hsp60 immunopositivity was very low or was under the detection threshold, with a mean positivity of 5.1% of the epithelial cells (Fig. 1C). The difference in the percentage of Hsp60-positive cells between tumor samples and normal controls was statistically significant. Moreover, statistical analyses revealed a correlation between both pT and pN parameters and Hsp60 IHC levels (Table 4). However, we did not find any correlation between Hsp60 IHC levels and tumor grade. Overall, these data are in agreement with previous results obtained by our group suggesting that Hsp60 levels are related to tumor progression but not to tumor grade, and which were subsequently
Figure 1. Heat shock protein 60 (Hsp60) gene expression and protein levels were increased in the affected intestinal tissue in patients with colon cancer compared with normal controls. (A) Quantitative real-time polymerase chain reaction demonstrated a higher expression of the hsp60 gene in adenocarcinomas (AC) compared with normal mucosa (NM). This difference was statistically significant as indicated by the asterisk in the histogram. (B) Western blot analysis demonstrated higher levels of Hsp60 in AC compared with NM. This difference was statistically significant as indicated by the asterisk in the histogram. (C) Immunohistochemical data were in agreement with the results of Western blot analysis in that Hsp60 levels were found to be higher in AC compared with NM. This difference was statistically significant as indicated by the asterisk in the histogram. Two representative photomicrographs of immunohistochemistry are shown on the right of the figure. Bar represents 100 μm. 2^-ΔΔCT indicates the Schmitgen and Livak calculation method.

TABLE 4. Analysis of Variance Between Hsp60 Immunohistochemical Levels in Tumor Samples and Clinical Variables

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adjusted Mean</th>
<th>Adjusted MS</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grading</td>
<td>2</td>
<td>25.97</td>
<td>12.99</td>
<td>0.79</td>
<td>.458</td>
</tr>
<tr>
<td>T classification</td>
<td>3</td>
<td>152.64</td>
<td>50.88</td>
<td>3.11</td>
<td>.035</td>
</tr>
<tr>
<td>N classification</td>
<td>2</td>
<td>184.81</td>
<td>92.26</td>
<td>5.64</td>
<td>.006</td>
</tr>
</tbody>
</table>

Abbreviations: DF, degrees of freedom; F value, Fisher distribution value; MS, mean square; SS, sum of square.

confirmed by other groups in smaller series of patients, indicating that Hsp60 is a very promising IHC marker for detecting large bowel cancer cells, thereby helping the pathologist in staging large bowel tumors.

Although in the tumor samples examined herein Hsp60 was present mainly in tumor cells, it also was immunopositive, although to a lesser extent, in the peritumoral interstitial space.

Hsp60 Intracellular Distribution in Tumor Cells
IEM demonstrated positivity for Hsp60 in tumor cells not only in its canonical location (mitochondria) but also in extramitochondrial sites such as cytosol and the plasma membrane (Figs. 2A-2D). By contrast, normal cells from control samples demonstrated scarce Hsp60 positivity that was confined to the mitochondria (Fig. 2E).

Hsp60 Distribution in Peritumoral Tissue
DIF demonstrated the presence of Hsp60 at the plasma membrane level in tumor cells (Fig. 3 top four panels). Moreover, DIF demonstrated Hsp60 positivity not only in tumor cells but also in peritumoral inflammatory cells such as those positive for CD57 (a marker for NK cells) (Fig. 3)
middle row of panels) and CD68 (a marker for macrophages) (Fig. 3 bottom row of panels).

**Presence, Levels, and Localization of Hsp60 in Exosomes**

Data regarding exosomal fraction assessment by standard methods (TEM, AChEase assay, and WB analysis for heat shock cognate 70 [Hsc70] and ALG-2-interacting protein X) to identify exosomes confirmed their identity.

In particular, TEM (Supporting Information Fig. 1A) demonstrated the typical exosomal-like vesicles (which we called "exosomes," in accordance with the literature) obtained from all groups studied (i.e., patients before surgery, patients after surgery, and healthy controls). In addition, the results obtained with the AChEase assay and the measurements of Hsc70 and Alix protein content by WB analysis also were typical of exosomes (Supporting Information Fig. 1B and 1C).
Hsp60 was present in the exosomes, but at different levels in patients before surgery and after surgery (Fig. 4A). The levels of Hsp60 in the exosomes of patients before surgery were significantly higher than in the exosomes from the same patients after surgery, among whom Hsp60 decreased to levels comparable to those of controls. ELISA confirmed the normalization of Hsp60 levels in the blood of patients after surgery (Fig. 4B). Carbonate tests demonstrated that Hsp60 is localized to the membrane of exosomes obtained from patients before surgery (Fig. 4C). By contrast, no Hsp60 was detected in the membrane of exosomes obtained from the same patients after surgery and from healthy controls (not shown).

**DISCUSSION**

The role of Hsp60 as a biomarker and pathogenic factor in cancer has been under scrutiny during the last few years. This chaperonin is emerging as a potentially useful tool for diagnosis and the assessment of prognosis and response to treatment, and also as a therapeutic agent or target. In the past, we demonstrated that Hsp60 is increased in preneoplastic lesions of the large bowel, including tubular adenomas with dysplasia and some forms of inflammatory bowel disease. Moreover, we demonstrated Hsp60 secretion by tumor cells, and have partially elucidated the mechanisms involved in secretion, in vitro, using cell lines. In the current study, we present what to our knowledge are the first data regarding Hsp60 distribution in tumor and peritumoral cells of colon cancer in vivo, as well as data regarding the presence, levels, and distribution of exosomal Hsp60 in blood samples obtained from patients with cancer before and after ablative surgery.

Our previous observations, made in vitro, demonstrated that the odyssey of Hsp60 from inside tumor cells to the outside involves the plasma membrane and exosomes. Data from the current study suggested that the same mechanisms might be operative in patients.
Moreover, in our previous article, we postulated that Hsp60 released by tumor cells through exosomes could interact with peritumoral cells as well as reach the bloodstream. In the current study, we found that among patients with colon cancer, Hsp60 is present in the pericellular interstitium of the affected tissue, localizes on macrophages and NK cells, and concomitantly occurs in the blood of the patients. All these new findings lend further support to the idea that Hsp60 in exosomes plays a critical role in the pathophysiology of colon cancer.

Very few studies to date have been performed in vivo regarding the presence of Hsp in exosomes obtained from patients with cancer, and to the best of our knowledge, changes in exosomal Hsp levels before and after surgery have not been described previously. The latter measurements of exosomal Hsp levels before and after surgical ablation of a tumor are a promising approach because the results are likely to be useful for early diagnosis and patient follow-up. For example, assessment of exosomal Hsp60 levels in tumor tissue and blood might be useful in the identification of high-risk patients and in the follow-up of patients treated with surgery to detect disease recurrence at an early stage. Because the use of exosomes in cancer therapy has been postulated, and Hsp60 has been found to be changed with regard to both quantity and distribution in tumors, the data presented herein encourage the belief that this chaperonin is a candidate for theranostics.

The term "theranostics" was coined to define ongoing efforts in clinics to develop more specific individualized therapies for various diseases, and to combine diagnostic and therapeutic capabilities into a single agent. In view of
this, and considering the findings of the current study and those of other investigators, it may be said that Hsp60 appears to be a good candidate for the typical theranostic molecule, and is useful for diagnosis, the assessment of prognosis and treatments, and as a therapeutic agent for the patient who produces it. Indeed, positivity for Hsp60 in both macrophages and, above all, NK cells suggests that Hsp60 might induce an effective antitumor response in vivo. If these observations are confirmed in other series of patients similar to that reported herein, and in experimental animals, the initiation of clinical trials could be justified to determine whether stimulation of host immune cells by Hsp60-bearing exosomes will improve patient survival.

The results of the current study support the following clinicopathologic projections. First, Hsp60 is a promising diagnostic marker for colon adenocarcinoma from the viewpoints of both histology and molecular pathology. The finding of increased hsp60 gene expression (mRNA) and protein levels suggests that Hsp60 may indeed be considered a useful candidate in the clinical management of patients with colorectal cancer. This conclusion, which was derived from the current study on what to our knowledge is a larger series of patients than reported previously, is also supported by earlier data from our group[29,30] and from others.[31,32] It is interesting to note that the Hsp60 protein levels appeared higher than expected from the observed corresponding mRNA levels. However, it is known that the correlation between gene expression and protein levels is usually poor. The reasons for this are varied, and include that gene expression and protein levels are regulated by different mechanisms that are largely independent of each other. The degradation rate of mRNA can be faster than that of the corresponding protein. Likewise, the transcription rate can be slower than the translation rate. There are instances in which transcription and protein levels correlate but this does not appear to be the case in the current study samples. Herein, with the information at hand, it is difficult to draw conclusions but one may consider as a working hypothesis that the rate of protein degradation is slowed down by posttranslational modifications, which would lead to a disproportionately high accumulation of protein with regard to mRNA levels. We are currently investigating this point. Second, Hsp60 intracellular redistribution in tumor cells involves the plasma membrane. We have already demonstrated in vitro that localization of Hsp60 in tumor cells in the plasma membrane accompanies its secretion by exosomes.[17] The finding of Hsp60 in tumor cell membrane and in exosomes obtained from the same subjects, which we believe are reported here for the first time, suggests that the same pathway of secretion observed previously in cell lines in vitro might occur also in vivo. This observation led us to exclude the treatment with anti-Hsp60 monoclonal antibody in those patients in the current study with colon cancer. Indeed, Hsp60 redistribution from tumor plasma membrane to multivesicular bodies (and, in turn, its secretion by exosomes) indicates that Hsp60 is not stably present on the tumor cell surface and therefore cannot be considered a useful target for these antibodies. Third, Hsp60 is present not only in tumor cells but also in peritumoral macrophages and NK cells. It is well known that macrophages have receptors for Hsp60 on their surface and the binding of Hsp60 to these receptors leads to the secretion of cytokines, including those recruiting NK cells.[18,19] Hence, Hsp60 in the peritumoral environment may stimulate an effective antitumor response by a 2-step mechanism. The first step would consist of the stimulation of macrophages to secrete cytokines that recruit NK cells and the second step would be the triggering of an effective NK cell antitumor response. A similar mechanism has been shown for Hsp70.[28] Fourth, the normalization of Hsp60 levels in patients after surgery suggests that if disease recurrence occurs, Hsp60 levels will increase again; in this case, we do not doubt the chaperonin will be a good candidate as a biomarker of disease status during the follow-up period.

For all these reasons, we believe that exosomal Hsp60 can become a novel tool in colon cancer theranostics, as already postulated for other exosomal markers in other tumors.[35] Analogously, its use should also be explored in other types of cancer. As a rule, treatment with exosomes should not be substituted for the other therapeutic strategies currently in use but it has been proposed as a supplementary therapy because exosomes are naturally produced nanovesicles and their reinfusion is expected to be safe and without any side effects noted among patients (except the risk of contamination of samples during the cell culture and isolation procedures).

The results of the current study point to Hsp60 as a promising focus for theranostics of colorectal adenocarcinoma and, possibly, other types of cancer as well. Likewise, the data open the door to a number of investigations pertaining to diagnosis and patient management, including the molecular mechanisms involved in exosome action and the participation of Hsp60 in these mechanisms.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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