HMGB1 and its hyper-acetylated isoform are sensitive and specific serum biomarkers to detect asbestos exposure and to identify mesothelioma patients

Andrea Napolitano¹,²,*; Daniel J. Antoine³,*; Laura Pellegrini¹; Francine Baumann¹; Ian Pagano¹; Sandra Pastorino¹; Chandra M. Goparaju⁴; Kirill Prokrym⁴; Claudia Canino⁴; Harvey I. Pass⁴; Michele Carbone¹; Haining Yang¹

1. University of Hawai‘i Cancer Center, 701 Ilalo Street, Honolulu, HI, 96813, USA
2. Department of Molecular Biosciences and Bioengineering, University of Hawai‘i at Mānoa, 1955 East-West Road, Honolulu, HI, 96822, USA
3. MRC Centre for Drug Safety Science, Department of Molecular & Clinical Pharmacology, University of Liverpool, Ashton Street, Liverpool, L69 3GE, UK
4. Department of Cardiothoracic Surgery, New York University, NYU Langone Medical Center, 560 First Avenue, New York, NY, 10016, USA

* A. N. and D. J. A. contributed equally to this work.

Running Title: HMGB1 isoforms in asbestos exposure and mesothelioma
**Corresponding Authors:** Haining Yang, University of Hawai‘i Cancer Center, 701 Ilalo Street, Honolulu, HI, 96813, USA. Phone: (808) 440-4588. Email: hyang@cc.hawaii.edu, correspondence related to HMGB1; Michele Carbone, University of Hawai‘i Cancer Center, 701 Ilalo Street, Honolulu, HI, 96813, USA. Phone: (808) 440-4596. Email: mcarbone@cc.hawaii.edu, correspondence related to mesothelioma; and Harvey I Pass, Department of Cardiothoracic Surgery, New York University, NYU Langone Medical Center, 560 First Avenue, New York, NY, 10016, USA. Phone: (212) 263-5969 Email: Harvey.Pass@nyumc.org, correspondence related to biomarkers other than HMGB1.

**Keywords:** HMGB1, mesothelioma, asbestos, biomarker

**Financial Support.** This study was supported by the NCI-R01 CA160715, DOD CA120355, The Riviera United 4-a Cure, and Mesothelioma Applied Research Foundation and Shino-Test Corporation to H.Y; by the V-Foundation to M.C and H.Y, by the NCI R01106567 to M.C., and by the University of Hawai‘i Foundation, which received donations to support mesothelioma research from Honeywell International Inc., to M.C.; by the EDRN NCI grant 5U01CA111295-08 and donations to support mesothelioma research from Belluck and Fox, and the Stephen E. Banner Foundation to H.P.; by the Wellcome Trust and the Medical Research Council to D.J.A.

**Conflict of Interest.** The University of Hawaii has filed for patents on HMGB1 and mesothelioma, on which H. Yang, M. Carbone, and H.I. Pass are named as inventors.
D.J. Antoine is listed as an inventor on granted patents related to the detection and measurement of HMGB1 and its isoforms (US patient number: US8748109 B2 (June 2014), European patient number: EP2449378 (March 2015), Japanese patient number: 5721707 (April 2015))
Translational Relevance

Over 20 million people in the US and many more worldwide have been exposed to asbestos, the main cause of malignant mesothelioma (MM). However, serological biomarkers to identify among potentially exposed cohorts those who have actually been exposed and thus are at risk for MM, and among them those who are developing MM suffer from relatively poor sensitivity and specificity. We report that total serum HMGB1 is sensitive and specific in differentiating individuals professionally exposed to asbestos from unexposed healthy controls. Moreover, we discovered that a specific HMGB1 isoform, hyper-acetylated HMGB1, accurately discriminates MM patients from asbestos-exposed individuals with 100% specificity and sensitivity. Our findings open novel opportunities to identify asbestos-exposed individuals and among them those who have developed MM.
ABSTRACT

Purpose: To determine whether serum levels of High Mobility Group Box Protein-1 (HMGB1) could differentiate malignant mesothelioma (MM) patients, asbestos-exposed individuals, and unexposed controls.

Experimental Design: Hyper-acetylated and non-acetylated HMGB1 (together referred to as total HMGB1) were blindly measured in blood collected from MM patients (n=22), individuals with verified chronic asbestos exposure (n=20), patients with benign pleural effusions (n=13) or malignant pleural effusions not due to MM (n=25), and healthy controls (n=20). Blood levels of previously proposed MM biomarkers fibulin-3, mesothelin, and osteopontin were also measured in non-healthy individuals.

Results: HMGB1 serum levels reliably distinguished MM patients, asbestos-exposed individuals, and unexposed controls. Total HMGB1 was significantly higher in MM patients and asbestos-exposed individuals compared to healthy controls. Hyper-acetylated HMGB1 was significantly higher in MM patients compared to asbestos-exposed individuals and healthy controls, and did not vary with tumor stage. At the cut-off value of 2.00 ng/ml, the sensitivity and specificity of serum hyper-acetylated HMGB1 in differentiating MM patients from asbestos-exposed individuals and healthy controls was 100%, outperforming other previously proposed biomarkers. Combining HMGB1 and fibulin-3 provided increased sensitivity and specificity in differentiating MM patients from patients with cytologically benign or malignant non-MM pleural effusion.
Conclusions: Our results are significant and clinically relevant as they provide the first biomarker of asbestos exposure and indicate that hyper-acetylated HMGB1 is an accurate biomarker to differentiate MM patients from individuals occupationally exposed to asbestos and unexposed controls. A trial to independently validate these findings will start soon.
INTRODUCTION

Malignant mesothelioma (MM) is an aggressive cancer associated with exposure to asbestos and other carcinogenic mineral fibers (1, 2). Asbestos is a generic name that identifies six naturally occurring silicate mineral fibers that were, and some of them still are, used commercially (3). In the US, the use of asbestos increased dramatically during World War II and it peaked in the late 70s, when over 700 tons of asbestos were imported. Asbestos use decreased considerably in the 80s, following the report of the International Agency for Research on Cancer (IARC) that identified asbestos as a group-1 human carcinogen (4). Nevertheless, the use of asbestos continues to increase exponentially in many developing countries, where an epidemic of MM is expected in the coming decades (5). MM develops after a latency of 20-60 years from asbestos exposure (2). Accordingly, in the US the incidence of MM has risen from less than 10 cases/year in the early 1950s, to about 3200 cases/year in 2004, and it has remained stable since then (1). In the US, MM continues to occur in former asbestos workers and in settings of prolonged indoor exposure to asbestos fibers (2). Moreover, with the increased development of rural areas, MM has been associated with environmental exposure to asbestos (6) and to other carcinogenic mineral fibers, such as erionite, wincherite, richterite (7), and antigorite (8), which were not included in the original list of mineral fibers that were called “asbestos” and whose use is not regulated by federal agencies (2, 3). Moreover, it was discovered recently that germline mutations of the BRCA1-associated protein 1 (BAP1) gene predispose to MM (9, 10) and decrease the threshold amount of asbestos required to induce MM (11).
Patients with pleural MM represent about 80% of MMs (2); they have a median survival of less than one year from diagnosis (1, 2). Early MM detection is associated with better response to therapy and a median survival of three years (12). Less than 5% of MM patients are identified at an early stage, as the development of clinical symptoms usually occurs when the disease is advanced. Several MM biomarkers have been investigated, including mesothelin (13, 14), osteopontin (OPN) (15) and fibulin-3 (16). However, there are concerns regarding their sensitivity, specificity or reproducibility (17-19). Presently, there are no biomarkers to identify individuals at higher risk of developing MM because of asbestos exposure, although preliminary evidence from our laboratory suggested that serum levels of High Mobility Group Box 1 protein (HMGB1) may increase following asbestos exposure and MM development (2, 20).

We reported that HMGB1, a damage-associated molecular pattern protein, contributes to asbestos carcinogenesis (21). When asbestos fibers lodge in the pleura, mesothelial cells and macrophages react to its presence attempting a phagocytic response. During this process, human mesothelial cells (HM) undergo programmed cell necrosis and release HMGB1 into the extracellular space (21). Extracellular HMGB1 in turn attracts more macrophages (22), creating a self-propagating inflammatory vicious cycle as asbestos fibers lodge in tissues for years. This chronic inflammatory process is associated with the release of HMGB1 and mutagenic radicals and may lead to MM (23, 24). MM cells that grow out of an HMGB1 rich environment are often “addicted” to HMGB1, as they actively secrete HMGB1 and require it to sustain their own growth (2, 20).
HMGB1 localization and secretion is regulated by acetylation of the lysine residues in its two nuclear localization signals (NLS1/2). Non-acetylated HMGB1 is mostly localized in the nucleus, where it binds to chromatin and stabilizes nucleosomes. When HMGB1 is hyper-acetylated, it is sequestered into the cytoplasm followed by active secretion into the extra-cellular space (25, 26).

Therefore, we hypothesized that 1) the HMGB1 secreted by MM cells was hyper-acetylated and that this isoform would be found in the sera of MM patients; 2) the HMGB1 released when HM die following asbestos exposure was the passively released non-acetylated HMGB1 isoform, and that this isoform would be present in the sera of asbestos-exposed individuals without MM.

To test our hypothesis, we analyzed hyper-acetylated and non-acetylated HMGB1 in the supernatant of MM cell lines and primary HM cultures with or without exposure to asbestos, and in the serum samples from MM patients and from individuals with a strong occupational history of asbestos exposure, and from unexposed healthy individuals as controls. We also assessed whether serum levels of HMGB1 and its hyper-acetylated isoform would help discriminate MM patients from patients with benign or malignant non-MM pleural effusions. Moreover, we compared the diagnostic accuracy of total and hyper-acetylated HMGB1 to other proposed MM biomarkers including fibulin-3, mesothelin and OPN.

MATERIALS AND METHODS

In vitro studies
HM were established in tissue culture from pleural fluid of patients with congestive heart failure and characterized via immunohistochemistry as previously described (27). HM in tissue culture were treated with crocidolite asbestos fibers, and tested between cell culture passages 2-5 (27). MM cell lines REN (28) and HP3 (29) –also referred to as Phi in our previous study (20)– were obtained respectively from Dr. Steven M. Albelda and Dr. Harvey I. Pass within the last 3 years and have been previously characterized (20, 28, 29). Short-tandem repeat (STR) analysis was performed on these lines upon arrival. Reauthentication of the cell lines confirming cell identity was performed after completion of experiments in July 2015 by the company Genetica DNA Laboratories. Testing for Mycoplasma contamination was performed monthly using LookOut® PCR Detection Kit (Sigma-Aldrich, St. Louis, MO). Cells were cultured for 48 hours in serum-free media. Supernatants were concentrated 50X before analysis.

**Study Population**

Full description of participants’ demographic characteristics is included in Table S1. Briefly, we tested the levels of HMGB1 isoforms and other MM biomarkers respectively in serum and plasma from 60 patients who presented to the emergency room with pleural effusions: 13 of them were diagnosed with a benign pleural effusion; 22 of them were diagnosed with MM; and 25 of them with a malignant non-MM pleural effusion (all diagnoses were confirmed by cytology and, for the malignant effusions by histopathology and immunohistochemistry). In addition, we studied serum and plasma from insulation workers from New York City, US, part of the “Selikoff cohort”, with a strong history of occupational exposure to asbestos (30) (n = 20); apparently healthy controls with no known history of asbestos exposure (n = 20) from Liverpool, UK. MM
patients and patients with other effusions were recruited in a hospital setting in New York City, US from 2005 through 2013. Patients were enrolled, after giving consent, for participation in the approved Institutional Review Boards and blood procurement protocols. The samples analyzed were from non-consecutive randomly selected participants/patients, and collected prior to any treatment. The number of individuals recruited in each group (minimum of 20) ensured an 80% power to detect an effect size (Cohen’s d) of 1.0, with α = 0.05.

**Mass Spectrometry**

HMGB1 isoforms present in our cell supernatants and in the human sera were blindly analyzed at the University of Liverpool by whole protein ESI-LC-MS. Post-translational modifications were confirmed by tandem mass spectrometry (LC-MS/MS), as described (31-33). Briefly, prior to MS analysis, samples (50 μl of cell supernatants or 200 μl of human serum) were immunoprecipitated with 5 μg of rabbit anti-HMGB1 antibody (ab18256, Abcam, Cambridge, UK), and then subjected to trypsin (Promega, Madison, WI) or GluC (New England Biolabs, Ipswich, MA) digestion according to manufacturer’s instructions, and de-salted using ZipTip C18 pipette tips (EMD Millipore, Billerica, MA). Assay validation data showing the robustness of the protocol have already been extensively published elsewhere (32). As a further control, total HMGB1 was also measured on the human samples using a commercially available ELISA kit (IBL International, Hamburg, Germany). Plasma levels of fibulin-3 (USCN, Wuhan, PRC), mesothelin (R&D, Minneapolis, MN), and OPN (R&D, Minneapolis, MN) were measured in MM patients and asbestos-exposed individuals with indicated commercially available ELISA kits following manufactures’ instructions. To measure plasma mesothelin we did
not use the FDA-approved Soluble mesothelin related peptides (0.4, 0.3-0.6 ng/ml) Mesomark® kit (Fujirebio Diagnostics Inc., Malvern, PA), which is not available for research purposes in the US, but a different commercially available ELISA kit (R&D, Minneapolis, MN). In our hands, testing a subset of samples from this cohort, there is a very good correlation between the two kits ($R^2 = 0.75$, $P < 0.0001$) (Figure S1A).

**Statistical Analysis**

Using Bartlett test, all the tested biomarkers presented significant heterogeneous variances between groups. Therefore, significance of two-groups comparisons was calculated using Mann-Whitney non-parametric test. $P$ values < 0.05 were considered significant. Results were expressed as median, 1st quartile – 3rd quartile. Sensitivities, specificities, and receiver operating characteristic (ROC) area under the curve (AUC) were calculated to evaluate each biomarker (total and hyper-acetylated HMGB1, fibulin-3, mesothelin, OPN) as well as biomarker composite scores. The classification variables that we considered were healthy controls, asbestos-exposed individuals, MM patients, patients with benign pleural effusions, and patients with malignant pleural effusions due to other causes. The test variables were the different biomarkers and biomarker composites. The analyses were conducted using GraphPad Prism 6.00 (GraphPad Software, La Jolla, CA). Biomarker composite scores were calculated by logistic regression from standardized biomarker values (17) to discriminate MM patients from patients with pleural effusions due to other causes. Logistic regressions were run using Stata 12 (StataCorp LP, College Station, TX). Whenever present, cut-off values corresponded to the Youden’s J index (i.e., highest sum of sensitivity and specificity minus one) (34).
RESULTS

Initially, we evaluated HMGB1 from concentrated supernatant of HM and MM cells in tissue culture. Unexposed HM did not release detectable HMGB1 into the extracellular space (Figure 1). When HM are exposed to 5 μg/cm² of crocidolite asbestos, ~60-70% of them undergo programmed necrosis within 48 hours (27). Accordingly, in the supernatant of HM exposed to asbestos (Asb-HM), we consistently detected high levels of non-acetylated HMGB1, as expected from cells undergoing necrosis (Figure 1A, C). Instead, in the supernatant of MM cells, we detected hyper-acetylated HMGB1, consistent with active secretion, as well as non-acetylated HMGB1 (Figure 1B, C), likely released by a fraction of MM cells undergoing necrosis, as they are grown in serum-free tissue culture condition. Altogether, supernatants from MM cells showed higher levels of total (non-acetylated + hyper-acetylated) HMGB1 with a prevalence of the hyper-acetylated isoform, compared to the supernatants from HM exposed to asbestos that contained prevalently the non-acetylated isoform (Figure 1C). The presence of non-acetylated HMGB1 in asbestos-exposed HM was associated with significant cell death with loss of the classic HM cobblestone appearance (Figure 1D). The differences in levels of non-acetylated and hyper-acetylated HMGB1 observed in tissue cultures supported our hypothesis and prompted us to study HMGB1 in human sera.

We compared the levels of total HMGB1 and its isoforms in the sera from 1) 20 unexposed healthy controls, 2) 20 insulators workers (30) that included individuals with 10 or more years of occupational asbestos exposure, who did not have pleural effusion or evidence of any malignancy at the time of sera collection, and 3) 22 MM patients who
had been diagnosed following the development of pleural effusion, a common presentation in MM.

In the serum samples from unexposed healthy controls, total levels of HMGB1 detected by MS were very low (1.4, 0.8-2.2 ng/ml), consistent with previously published HMGB1 values in healthy volunteers (32). Total HMGB1 serum levels were significantly higher in asbestos-exposed individuals (10.2, 5.7-12.1 ng/ml) compared to the levels in unexposed controls \( (P < 0.001) \). MM patients had the highest levels of total HMGB1 (25.0, 15.7-36.6 ng/ml) when compared to either other group \( (P < 0.001) \) (Figure 2A). The total levels of HMGB1 measured with a commercially available ELISA kit and with our MS protocol were very similar \( (R^2 = 0.92, P < 0.0001) \) (Figure S1B), corroborating the reliability of our approach. The levels of hyper-acetylated HMGB1 were very low in both the healthy controls (0.5, 0.3-0.7 ng/ml) and asbestos-exposed individuals (0.4, 0.3-0.6 ng/ml). MM patients showed significantly higher levels of hyper-acetylated HMGB1 (17.4, 10.3-21.9 ng/ml) compared to either other group \( (P < 0.001) \) (Figure 2B). Overall, hyper-acetylated HMGB1 comprised ~10% of the total HMGB1 in the sera of asbestos-exposed individuals, and ~67% of the total HMGB1 in the sera of MM patients (Figure S2).

Next, we evaluated the sensitivity and specificity of total and hyper-acetylated HMGB1 as potential biomarkers to discriminate MM patients from asbestos-exposed individuals and healthy controls. Both, total and hyper-acetylated HMGB1, showed exceptional accuracy in discriminating MM patients from healthy controls with a AUC of 0.999 (95% CI 0.994-1.000) and 1.000 (95% CI 1.000-1.000), respectively. Comparing asbestos-exposed individuals to healthy controls, the AUC of total and hyper-acetylated HMGB1
were 0.964 (95% CI 0.893-1.000) and 0.574 (95% CI 0.392-0.756), respectively (Table 1 and Figure S3). These data suggest that total HMGB1 is a reliable biomarker to discriminate individuals with asbestos-exposure and/or MM from healthy controls. Comparing MM patients and asbestos-exposed individuals, the AUC of total HMGB1 was 0.830 (95% CI 0.687-0.972) (Figure 2C); at specificity 100%, the sensitivity was 72.73% (for values > 15.75 ng/ml, which also corresponded to the cut-off value); at sensitivity 100%, the specificity was 5%. The AUC of hyper-acetylated HMGB1, when comparing MM patients to asbestos-exposed individuals, was 1.000 (95% CI 1.000-1.000) (Figure 2D), with cut-off value of 2.00 ng/ml. These results point to hyper-acetylated HMGB1 as a novel, sensitive and specific biomarker to discriminate MM patients from asbestos-exposed individuals. We did not detect any significant difference in total or hyper-acetylated HMGB1 serum levels in MM patients with stage I-II vs. III-IV (Figure 2E, F), suggesting that early lesions are also associated with increased HMGB1 levels and that hyper-acetylated HMGB1 may be a valuable screening tool for early detection of MM among asbestos-exposed cohorts.

Next we sought to determine whether total and hyper-acetylated HMGB1 could also help differentiating MM patients from patients with pleural effusions due to other cause. Thirteen serum samples from patients with cytologically benign pleural effusions and 25 serum samples from patients with pleural effusions due to non-MM malignancy were available for these studies (Table S1). We found that MM patients had significantly higher levels of total HMGB1 compared to patients with cytologically benign pleural effusions (6.4, 4.7-9.7 ng/ml; \( P < 0.001 \)) and malignant (non-MM) pleural effusions (6.7, 4.2-10.0 ng/ml; \( P < 0.001 \)) (Figure 3A). Similarly, levels of hyper-acetylated HMGB1
were significantly higher in the sera from MM patients compared to sera from patients with benign pleural effusions (5.2, 3.7-7.8 ng/ml; \( P < 0.001 \)) or malignant (non-MM) pleural effusions (5.7, 3.3-8.2 ng/ml; \( P < 0.001 \)) (Figure 3B). Next, we evaluated the sensitivity and specificity of total and hyper-acetylated HMGB1 to discriminate MM patients from patients with pleural effusions due to other cause. The AUC of total HMGB1 was 0.860 (95% CI 0.736-0.984) (Figure 3C); at specificity 100%, the sensitivity was 63.64%; at sensitivity 100%, the specificity was 10.53%. The AUC for hyper-acetylated HMGB1 was 0.837 (95% CI 0.709-0.966) (Figure 3D); at specificity 100%, the sensitivity was 59.09%; at sensitivity 100%, the specificity was 10.53%. Best cut-off values to discriminate MM patients from patients with benign or malignant non-MM pleural effusions were 11.35 ng/ml (sensitivity 81.82%, specificity 89.47%) and 9.70 ng/ml (sensitivity 77.27%, specificity 89.47%) respectively for total and hyper-acetylated HMGB1. Overall, levels of total and hyper-acetylated HMGB1 were helpful to distinguish MM patients from patients with pleural effusions due to other cause.

The demographics of our cohorts can be found in Table S1. Very low levels of hyper-acetylated HMGB1 (< 2.00 ng/ml) were found both in asbestos-exposed individuals and healthy controls. We speculate that this basal hyper-acetylated HMGB1 might be involved in the process of physiological immune surveillance. We performed univariate analyses to identify factors that could influence HMGB1 levels (Table S2). Due to the small sample size, a reliable multivariate model (\( R^2 > 0.50 \)) to explain the total and hyper-acetylated HMGB1 variations was not found.

We also measured the levels of three previously proposed MM biomarkers (fibulin-3, mesothelin, and OPN) from the same asbestos-exposed individuals and MM patients.
All three biomarkers were significantly higher in MM patients compared to the asbestos-exposed individuals ($P < 0.001$) (Figure 4A-C, Table S3). Fibulin-3 had AUC of 0.959 (95% CI 0.905-1.000), mesothelin had AUC of 0.934 (95% CI 0.858-1.000), and OPN had AUC of 0.961 (95% CI 0.910-1.000) (Figure 4D-F). However, none of these biomarkers reached the sensitivity and specificity achieved by hyper-acetylated HMGB1 (AUC of 1.000) to discriminate these two cohorts (Figure 2D).

When comparing MM patients to patients with pleural effusions due to other cause, the levels of fibulin-3 were significantly higher in MM patients vs. either benign pleural effusion or other malignant non-MM effusion ($P < 0.001$) (Figure 4G). Similarly, mesothelin levels were higher in MM patients vs. the benign effusion group ($P < 0.01$) or vs. the non-MM malignant effusion group ($P < 0.001$) (Figure 4H). Instead, OPN levels did not show significant difference among the groups (Figure 4I). Fibulin-3 had an AUC of 0.928 (95% CI 0.868-0.989), mesothelin had an AUC of 0.798 (95% CI 0.678-0.918), and OPN had an AUC of 0.502 (95% CI 0.348-0.657) (Figure 4J-L). Total HMGB1 (AUC = 0.860) and hyper-acetylated HMGB1 (AUC = 0.837), performed better than OPN and mesothelin in this comparison (Figure 3C-D and 4J-K).

Among MM patients, levels of total and hyper-acetylated HMGB1 did not correlate with any of the other biomarkers (Figure S4), suggesting independent roles of these molecules in MM. We therefore calculated combined values of biomarkers derived from logistic regression equations. AUC were calculated for those combinations of biomarkers with regression equations having pseudo-$R^2 > 0.75$ and all the parameters with $P < 0.05$. Combined values of fibulin-3 and either total or hyper-acetylated HMGB1 resulted in improved sensitivity and specificity in discriminating MM patients from
patients with pleural effusions due to other cause, with AUC of 0.987 (0.967-1.000) and 0.981 (0.956-1.000), respectively. Best cut-off values to discriminate MM patients from patients with benign or malignant non-MM pleural effusions were for scores of -0.48 (sensitivity 95.45%, specificity 92.11%) and -0.22 (sensitivity 90.91%, specificity 92.11%) respectively for fibulin-3 with either total or hyper-acetylated HMGB1 (Figure 5 and Table S4).

In summary, hyper-acetylated HMGB1 showed the highest AUC in discriminating MM patients from asbestos-exposed individuals. The combination of fibulin-3 and HMGB1 increased the power of discrimination of MM patients from patients with pleural effusions due to other cause.

**DISCUSSION**

We report that elevated serum levels of total HMGB1 differentiate asbestos-exposed individuals and MM patients from healthy unexposed controls. Moreover, we discovered that hyper-acetylated HMGB1 is a very sensitive and specific biomarker to discriminate MM patients from asbestos-exposed individuals and from healthy unexposed controls. These results were supported by *in vitro* experiments in which we found that asbestos-induced necrosis of HM leads to the passive release of non-acetylated HMGB1, whereas the supernatant of MM cells contains mostly hyper-acetylated HMGB1, which is the isoform that is actively secreted. We propose that HMGB1 and its hyper-acetylated isoform can be a valuable tool to identify among potentially exposed people, those individuals who have been exposed to asbestos, and to identify among them those who have developed MM.
The extremely high specificity and sensitivity (100%) of hyper-acetylated HMGB1 to identify MM patients from asbestos-exposed individuals (Figure 2D) exceeded our most optimistic expectations. In this setting, hyper-acetylated HMGB1 outperformed other previously proposed MM biomarkers, two of them, OPN and fibulin-3, identified by our co-author (H.I. Pass) (15, 16) who performed the comparative analyses shown in Figure 4.

We previously proposed total HMGB1 as a possible biomarker for asbestos exposure and MM (20, 21) but we did not investigate its possible value in differentiating MM patients from asbestos-exposed individuals. Here, we confirmed that the total serum levels of HMGB1 are elevated in both asbestos-exposed individuals and MM patients, and reliably distinguish these cohorts from unexposed healthy controls. We further discovered that MM patients have significantly higher total serum HMGB1 levels compared to asbestos-exposed individuals (P < 0.001). Although total HMGB1 levels are sensitive and specific to discriminate asbestos-exposed from unexposed individuals, the relatively low AUC of 0.830 when comparing MM patients with asbestos-exposed individuals would limit its clinical usefulness in identifying MM patients among large cohorts of asbestos-exposed individuals. Strikingly, hyper-acetylated HMGB1 reliably distinguished MM patients from individuals occupationally exposed to asbestos with 100% sensitivity and specificity. Identifying MM patients among the high-risk asbestos-exposed cohorts is extremely difficult, due to lack of early phenotypical evidences of MM. The specificity and sensitivity of hyper-acetylated HMGB1 should significantly facilitate this task.
We also measured the levels of various biomarkers in patients with benign or malignant non-MM pleural effusions, and compared them to MM patients (Table S3, Figures 3 and 4). We found that total and hyper-acetylated HMGB1 were second to fibulin-3 in distinguishing MM patients from patients with pleural effusions due to other cause, and performed better than mesothelin and OPN. However, the combination of fibulin-3 and HMGB1 resulted in even higher power of discrimination, suggesting a clinical advantage in measuring both proteins.

In our sample set, mesothelin and OPN performed better than in some previous studies (18, 19). Fibulin-3 data were consistent with the original report (16) and with additional studies (35, 36), and were better than those reported by other groups (37, 38). A possible partial explanation to this observation is that most circulating biomarkers are very sensitive to variability introduced by samples’ preparation and handling, and choice of adequate control groups. The problem of reproducibility in biomarker studies is very important, and standardization of protocols to collect and store samples is a necessary step that needs to be taken (39).

Of note, the serum samples from the exposed cohort tested here were from insulators with many years of exposure to high amounts of asbestos (30). Further studies are needed to analyze whether HMGB1 levels are increased also in a setting of exposure to lower amounts of asbestos. Also, it has been shown that total HMGB1 levels in serum and plasma are elevated in severe trauma and septic shock. However, these are transient increases that occur only in severe circumstances that require hospitalization in intensive care. Under these conditions, HMGB1 levels return to baseline within hours to 7 days (41-43). HMGB1 is also increased in patients with sepsis who succumbed to
the infection (44), and in some cases of chronic autoimmune disease (45). Specifically, patients with rheumatoid arthritis may have serum levels of HMGB1 above baseline (46, 47), although the levels are more frequently increased in the synovial fluid than in the serum (48). In contrast to all of the above, individuals heavily exposed to asbestos have sustained high HMGB1 serum levels – due to the bio-persistence of the asbestos fibers lodging in the tissues that cannot be removed, suggesting that serial longitudinal analysis of total HMGB1 will prove helpful to identify among potentially exposed cohorts those individuals who have actually been exposed and are at risk of developing MM. Close follow up of these high-risk individuals, and testing for hyper-acetylated HMGB1 in their serum, should help detect MM at an earlier stage when it is more susceptible to therapy. Notably, the levels of total and hyper-acetylated HMGB1 were not influenced by stage, suggesting that HMGB1 isoforms might be a promising early MM detection biomarker.

In vitro, HMGB1 can be hyper-acetylated and released by monocytes and macrophages (25). So far, among the non-malignant conditions, hyper-acetylated HMGB1 has only been detected in alcoholic liver disease (ALD) (31), acute acetaminophen-induced liver failure (32), and severe macrophage activation syndrome (33). In the latter two conditions, however, the increase of serum HMGB1 is transient and limited to the acute crisis when patients are in intensive care. ALD might represent a possible confounding factor. However, a specific phosphorylation at serine 34 has been identified in circulating HMGB1 from ALD patients (31). Instead, HMGB1 phosphorylation was not detected in any of our samples (data not shown), using the same analytical methodology of detection, and by the same investigator who performed the ALD study.
Therefore, mass spectrometry, by revealing the presence of phosphorylated HMGB1 isoforms, can reliably distinguish the hyper-acetylated HMGB1 isoform found in ALD patients from the hyper-acetylated HMGB1 isoform found in MM patients.

Potential technical limitations of our results need to be considered: our sample size was relatively small, which could lead to an undesired over-fitting of the data; our patients and controls were not strictly matched for age, sex, ethnicity, smoking status, or other demographic/epidemiologic factors, potentially introducing unwanted confounding factors. Before total and hyper-acetylated HMGB1 can be introduced into the clinic as biomarkers of asbestos exposure and MM detection, our findings need to be independently validated in a larger cohort. Prospective longitudinal validation studies with matched case-controls will start soon to validate the results reported here in a larger cohort with funding from the NCI-EDRN. In these studies, we will also simultaneously investigate whether HMGB1 levels can be affected, similar to mesothelin, by clinical covariates such as kidney function or body-mass-index (49), and whether potential correlations exist between HMGB1 isoforms and other known markers of chronic inflammation, such as C-reactive protein or neutrophil-to-lymphocyte ratio. Moreover, further studies will be performed to investigate the potential role of specific HMGB1 isoforms as markers of prognosis or tumor recurrence after surgical cytoreduction, and as therapeutic targets. Specific reagents able to detect HMGB1 isoforms in a hospital setting will have to be developed. In this regard, we are currently trying to develop ELISA assays for specific HMGB1 isoforms. Nevertheless, the
exceptional potential relevance of our findings to asbestos exposed individuals and MM patients warrants immediate attention.
REFERENCES


Table 1. AUC of total and hyper-acetylated HMGB1 comparing healthy controls, asbestos-exposed individuals, and MM patients.

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<th>Total HMGB1</th>
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<td>AUC (95% CI)</td>
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Figure Legends

Figure 1. Mass spectrometry analyses. Asbestos-exposed HM and MM cells release different HMGB1 isoforms. (A) Representative spectra of whole protein electro-spray ionization mass spectrometry of HMGB1 in crocidolite asbestos-exposed HM (Asb-HM): only non-acetylated HMGB1 is detected. (B) Representative spectra of whole protein electro-spray ionization mass spectrometry of HMGB1 in MM cells: both hyper-acetylated and non-acetylated HMGB1 are detectable. (C) Quantification of HMGB1 isoforms in supernatant of primary HM culture, Asb-HM, and MM cells. Results were reproducible in 3 primary HM derived from different patients, and 2 MM cell lines. (D) Microscope pictures (40X) of HM, Asb-HM, and MM cells showing respectively classical cobblestone morphology in normal culture (HM), or dying HM due to asbestos exposure (Asb-HM), and MM cells with spindle-like morphology (MM).

Figure 2. HMGB1 serum levels distinguished MM patients, asbestos-exposed individuals, and unexposed controls. (A-B) Quantification of total and hyper-acetylated HMGB1 in serum samples from 20 healthy volunteers (H), 20 individuals occupationally exposed to asbestos for at least 10 or more years (Asb), and 22 MM patients (MM). (A) Total HMGB1 serum levels are higher in asbestos-exposed individuals compared to healthy unexposed controls, and highest in MM patients; *** \( P < 0.001 \). (B) MM patients showed significantly higher serum levels of hyper-acetylated HMGB1 compared to either other group; *** \( P < 0.001 \). Horizontal lines at 15.75 ng/ml (A) and 2.00 ng/ml (B) show cut-off values that discriminate MM patients from asbestos-exposed individuals. (C-D) ROC curves of total HMGB1 (C) and hyper-acetylated HMGB1 (D) comparing MM patients to asbestos-exposed individuals. (E-F) Levels of
total and hyper-acetylated HMGB1 in patients with early or late stage of MM (stage I-II vs. stage II-IV). No statistical significant difference was found.

**Figure 3. Total and hyper-acetylated HMGB1 differentiate MM patients from individuals with pleural effusions due to other cause.** (A-B) Quantification of total and hyper-acetylated HMGB1 in MM patients, patients with cytologically benign pleural effusions (Ben-PE), and patients with cytologically malignant (non-MM) pleural effusions (Mal-PE). Both total and hyper-acetylated HMGB1 levels are higher in MM patients; *** \( P < 0.001 \). Horizontal lines at 11.35 ng/ml (A) and 9.70 ng/ml (B) show cut-off values that discriminate MM patients from patients with pleural effusion due to other cause. (C-D) ROC curves of total and hyper-acetylated HMGB1 comparing MM patients to patients with pleural effusions due to other cause.

**Figure 4. Performance of other proposed biomarkers: fibulin-3, mesothelin and OPN.** (A-C) Quantification of other proposed biomarkers in MM patients and asbestos-exposed individuals (Asb). The levels of all three biomarkers are higher in MM patients compared to asbestos-exposed individuals, *** \( P < 0.001 \). (D-F) ROC curves of fibulin-3, mesothelin, and OPN comparing MM patients to asbestos-exposed individuals. (G-I) Quantification of fibulin-3, mesothelin, and OPN in MM patients, patients with cytologically benign pleural effusions (Ben-PE), and patients with cytologically malignant (non-MM) pleural effusions (Mal-PE). Fibulin-3 and mesothelin can differentiate MM patients from patients with pleural effusions due to other cause; ** \( P < 0.01 \), *** \( P < 0.001 \). (J-L) ROC curves of fibulin-3, mesothelin, and OPN comparing MM patients and patients with pleural effusions due to other cause. Fibulin-3 has the highest AUC.
Figure 5. Combination of fibulin-3 and HMGB1 in distinguishing MM patients from patients with pleural effusions due to other cause. (A-B) Combined values derived from logistic regression analysis of fibulin-3 and either total or hyper-acetylated HMGB1 in MM patients and patients with benign pleural effusions (Ben-PE), and patients with cytologically malignant (non-MM) pleural effusions (Mal-PE). *** P < 0.001. Horizontal lines at scores of -0.48 (A) and -0.22 (B) show cut-off values that discriminate MM patients from patients with pleural effusions due to other cause. (C-D) ROC curves of fibulin-3 alone, HMGB1 (total or hyper-acetylated) alone, and combination of the previous showing increased AUC.
Figure 1

A

Asb-HM

NH₂ COOH: Non-acetylated HMGB1

B

MM

NH₂ COOH: Hyper-acetylated HMGB1

NH₂ Ac Ac COOH: Non-acetylated HMGB1

C

<table>
<thead>
<tr>
<th></th>
<th>Hyper-acetylated HMGB1</th>
<th>Non-acetylated HMGB1</th>
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<tr>
<td>HM</td>
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<tr>
<td>MM</td>
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</tr>
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</table>

D

HM

Asb-HM

MM
Figure 3

A. Total HMGB1

B. Hyper-acetylated HMGB1

C. Total HMGB1

D. Hyper-acetylated HMGB1

AUC = 0.860

AUC = 0.837
Figure 4

A. Fibulin-3 (ng/ml)

B. Mesothelin (ng/ml)

C. OPN (ng/ml)

D. Sensitivity %

Fibulin-3

E. Sensitivity %

Mesothelin

F. Sensitivity %

OPN

AUC = 0.959

AUC = 0.934

AUC = 0.961

G. Fibulin-3 (ng/ml)

H. Mesothelin (ng/ml)

I. OPN (ng/ml)

J. Fibulin-3

K. Mesothelin

L. OPN

AUC = 0.928

AUC = 0.798

AUC = 0.502
Figure 5

A

Fibulin-3 + Total HMGB1

B

Fibulin-3 + Hyper-acetylated HMGB1

C

100% - Specificity%

D

100% - Specificity%

- Fibulin-3
- Total HMGB1
- Fibulin-3 + Total HMGB1

- Fibulin-3
- Hyper-acetylated HMGB1
- Fibulin-3 + Hyper-acetylated HMGB1
Clinical Cancer Research

HMGB1 and its hyper-acetylated isoform are sensitive and specific serum biomarkers to detect asbestos exposure and to identify mesothelioma patients

Andrea Napolitano, Daniel J Antoine, Laura Pellegrini, et al.

Clin Cancer Res  Published OnlineFirst January 5, 2016.

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