Microparticles and Malignant pleurisy

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CHAPTER 1: PLEURAL EFFUSION

1.1 PHYSIOLOGIC TURNOVER OF PLEURAL EFFUSION

The pleural space is bounded by the parietal and visceral membranes covered by a continuous layer of pleural mesothelial cells. Studies of pleural liquid dynamics in the normal pleural space are limited. Available data indicate that pleural fluid is formed from the systemic vessels of the pleural membranes at an approximate rate of 0.6 mL/h and is absorbed at a similar rate by the parietal pleural lymphatic system. Normally, the pleural spaces contain approximately 0.25 mL/kg of low protein liquid. Disturbances in either formation or absorption result in the accumulation of excess pleural fluid\(^1\).

1.1.1 Pleural anatomy

The pleural space is a real, not potential, space that is approximately 10 to 20 micrometres wide and extends completely around the lung to the hilar root\(^2\)\(^3\)\(^4\). The visceral pleura covers the lung and interlobar fissures; the parietal pleura covers the chest wall, diaphragm, and mediastinum. Each pleural interface has a surface area of approximately 1000 cm. Each membrane is covered by a single cell layer of mesothelial cells and each (at least in humans and large mammals) is supplied by a systemic circulation.

The visceral and parietal pleural membranes differ in one important respect: only the parietal pleura has lymphatic stomata that open directly into the pleural space. Current concepts of pleural liquid turnover have stemmed in large part from considering the differential anatomy of the two pleural membranes\(^5\).

Parietal pleura

The parietal pleura has been proposed as the more important pleura for pleural liquid turnover in the normal physiologic state. The reasons for this hypothesis lie in the proximity of the microvessels to the pleural space, the presence of the lymphatic stomata, and the consistent anatomy of the parietal pleura among species. The parietal pleural membrane overlies the intercostal fascial layer and ribs. It is approximately 30 to 40 micrometres thick, consisting of
a superficial mesothelial layer and subpleural layer. Within the subpleural layer lies loose connective tissue in which run the intercostal arteries, nerves and lymphatics. The intercostal microvessels are about 10 to 12 micrometres from the pleural space.

**Figure 1: Pleural images at scanning electron-microscope photomicrograph**

a – Lymphatic stomata on the tendinous pleural diaphragmatic surface (modified from Negrini et al 1991); b - Semithin cross-section of rat diaphragm showing the arrangement of the diaphragmatic lymphatic network originating from the pleural (PL) and the peritoneal (PE) surfaces. Four types of lymphatic structures may be distinguished: submesothelial lacunae (arrowhead) within the interstitial space beneath the mesothelial layer; transverse ducts (arrow) running perpendicularly to the lacunae through the muscular fibres (M); central collectors (C), in the deep diaphragm; c – At transmission electron microscopy (TEM) the pleural submesothelial connective tissue layer is composed of loose collagen fibres (CF) organized in bundles and contains lymphatic lacunae lined by a discontinuous endothelium (arrowheads). ME, mesothelium; d - Transverse ducts (arrow) lined by a discontinuous endothelium and central collector vessels (C) lined by a continuous endothelium; e - Transverse lymphatic ducts with intraluminal valves formed by two leaflet (arrowheads) attached at opposite sides of the lymphatic vessel walls; f - Primary valves formed by two adjacent endothelial cells of a diaphragmatic initial lymphatic; b, c, d, e, f; modified from Grimaldi et al 2006)

(From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)

The most interesting and unusual features of the parietal pleura are the lymphatic stomata, holes of 2 to 6 micrometres in diameter that open onto the pleural space. These measurements were obtained in the resting state; the diameters likely increase with chest expansion during ventilation. The stomata have been demonstrated on the parietal pleural surface with scanning
electron microscopy. Each stoma is formed by a gap in the otherwise continuous mesothelial cell layer, where the mesothelial cells join with the endothelial cells of the lymphatics. Each lymphatic joins others, forming a lake or lacuna; from the lacunae, collecting lymphatics join intercostal trunk lymphatics, which travel to the parasternal and periaortic lymph nodes. The number of stomata over the caudal pleural surfaces (chest wall and mediastinum) in humans is not known with certainty. Estimates based on animal models suggest a range from 100 to 200 stomata per cm\(^2\). The distribution of stomata in some animals is nonuniform with a larger number of openings found in dependent regions\(^8\).

By microscopy studies, including electron microscopy, these stomata have been shown to connect directly to the pleural space and to accommodate intact erythrocytes or carbon particles previously injected into the pleural space. Using live imaging with videothoracoscopy in monkeys, researchers have been able to see carbon particles move from the pleural space into the lymphatics in the costal, mediastinal, and diaphragmatic pleura within 15 minutes and to drain to collecting lymphatics within 30 minutes\(^9\).

**Visceral pleura**

The visceral pleura in humans is approximately 20 to 80 micrometres thick and consists of a mesothelial layer and a subpleural connective tissue layer\(^10\). The thickness varies considerably over the lung, being greatest in the caudal regions and least in the cranial regions. The subpleural connective tissue layer contains both collagen and elastin, as well as the bronchial artery capillaries and subpleural lymphatics (which do not connect to the pleural space). The alveoli and the pulmonary circulation lie beneath the visceral pleural membrane. Both the bronchial microvessels and lymphatics are farther from the pleural space than in the parietal pleura (20 to 50 micrometres versus 10 to 12 micrometres, respectively).

The bronchial microvessels drain into the pulmonary veins, a feature that may have two consequences for pleural liquid formation:

Because of the normally low pulmonary venous pressure, the bronchial arterial driving pressure is probably lower than in other systemic microvessels, which have a systemic drainage. This may mean that, in the normal situation, less liquid flows from the visceral pleura than from the parietal pleura.

When the pulmonary vascular pressures rise, the bronchial arterial driving pressure and visceral pleural liquid flow could increase resulting in transvascular filtration; pleural liquid formation could increase as a result.

Interestingly, visceral pleural anatomy differs strikingly among species of mammals. In small mammals (mice, rabbits, dogs), the visceral pleura is quite thin (5 to 10 micrometres), with
almost no subpleural layer and no pleural bronchial circulation. One likely possibility for the major difference in anatomy is structural; in large mammals, the thick visceral pleura may offer necessary support for the lung tissues. The visceral pleural connective tissue may withstand and dissipate stresses in the lung, minimize overexpansion of weaker portions of lung (thereby reducing the risk of pneumothorax), and smooth out the relative expansion of different areas of lung and the distribution of ventilation.

**Pleural mesothelial cell**

The continuous lining cell of both pleurae is the mesothelial cell\(^3\). Not unique to the pleural space, the mesothelial cell lines the other two coelomic spaces in the body, the pericardial and peritoneal spaces. There are no differences yet described among mesothelial cells from these three locations or between the parietal and visceral locations. The mesothelial cell is a flat cell (1 to 4 micrometres thick) with a variable covering of microvilli (up to 3 micrometres long). The shape and area of the (visceral) mesothelial cell changes with lung inflation, with diameters that range from 27 micrometres at a transpulmonary pressure of 1.5 cm H\(_2\)O to 39 micrometres at 12 cm H\(_2\)O in isolated rabbit lungs\(^{11}\).

Similar to that of the other two lining cell types, i.e., the endothelial and epithelial cells, the mesothelial cell has been found to have many functions\(^{12}\)\(^{13}\). It produces a wide array of extracellular matrix molecules and may participate in the production of the submesothelial connective tissue\(^{14}\).

The mesothelial cell, at least in vitro, is phagocytic\(^{15}\)\(^{16}\). It may function as an inflammatory cell, directing movement of other inflammatory cells into the pleural space by releasing cytokines and expressing adhesion molecules\(^{17}\)\(^{18}\)\(^{19}\). The mesothelial cell may also recruit fibroblasts\(^{20}\).

Mesothelial cells may contribute to the balance between procoagulant and fibrinolytic activities in the pleural space\(^{21}\)\(^{22}\).

Compared to all these known or potential functions, however, the mesothelial cell has no documented active role in liquid entry or exit from the pleural space. There is no evidence for active transport by the mesothelial cell. Furthermore, the mesothelial surface is leaky to protein and liquid, as, for example, is necessary for successful dialysis in the peritoneal space. A leaky membrane implies a passive role for the mesothelium in the movement of liquid and protein.
Figure 2: Pressures on pleura
A: The hydraulic pressures in the costal region of the pleural space (Pliq, Miserocchi et al 1983) and in the parietal extrapleural (Pepi, Negrini et al 1987) and pulmonary (Ppi, Miserocchi et al 1993) interstitial spaces as a function of lung height in supine anesthetized rabbits and at end-expiratory lung volume (FRC). The pleural surface pressure, an index of region lung recoil is also reported for comparison (dotted line, Agostini 1986); B: The costal, mediastinal and diaphragmatic Pliq values at the end-expiratory (dashes lines) and end-inspiratory (continuous lines) lung volumes (Miserocchi et al 1981) (From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)

1.1.2 Pleural liquid formation

Much of what we know about normal pleural liquid turnover is derived from studies in sheep, which have a pleural anatomy similar to that in humans. Studies of normal liquid turnover have been hampered by the narrowness of the pleural space and its sensitivity to inflammation. Most experiments, therefore, have relied on non-invasive studies of liquid formation, with the assumption that, in a steady-state condition, liquid will be formed and absorbed at the same rate.

Normal pleural liquid

The volume of pleural liquid is small, approximately 0.1 to 0.2 mL per kg in different species. One study in normal humans found a mean pleural fluid volume of 8.4 mL per hemithorax, or 0.26 mL per kg total\textsuperscript{23}. The WBC count in this group of subjects was approximately 1700 per mm\textsuperscript{3}, with a median differential of approximately 75 percent macrophages and 23 percent...
lymphocytes. The normal protein concentration of the pleural liquid is low, approximately 15 percent of the plasma protein concentration.

**Rate of formation**

In non-invasive studies using equilibration of radiolabelled albumin from the plasma to the pleural liquid, pleural liquid in the sheep formed at 0.01 mL/kg per hour, or the equivalent of 0.6 mL/hour in a 60-kg person. This constituted a turnover rate of 11 percent of the pleural liquid volume per hour.

**Origin of pleural liquid**

The current consensus of pleural liquid formation is that the liquid originates from the systemic vessels of the pleural membranes, not from the pulmonary vessels. In other words, pleural liquid is interstitial fluid of the systemic pleural microvessels. There are three major considerations that support this hypothesis:

The systemic vessels (of both parietal and visceral pleural membranes) are adjacent to the pleural space and are much closer to the pleural space than are the pulmonary vessels.

The low pleural liquid protein concentration (1 g/dL) and ratio to the plasma protein concentration (0.15 g/dL) are consistent with a filtrate from high-pressure systemic vessels. If liquid and protein are filtered at high pressure and high flow across a semipermeable membrane, large particles will be sieved and relatively restrained compared to the liquid. Thus, plasma proteins, being large, will be retarded much more than the liquid in their movement across a membrane, and the protein concentration of the resultant filtrate will be low. On the other hand, if liquid and protein are filtered at low pressure and low flow, proteins are retarded less, and the protein concentration of the resultant filtrate is higher. Filtrates from low-pressure pulmonary vessels, e.g., lung lymph, have a high protein concentration (4.5 g/dL) and ratio (0.7) compared to filtrates from systemic vessels and to pleural liquid.

Of note in this argument, pleural liquid formation is described as high flow, whereas its measured rate is relatively slow (0.01 mL/kg per hour). However, it is the filtration at the systemic microvessels that is described as high, or at least higher than filtration across pulmonary microvessels. Some of that filtrate is reabsorbed into the low-pressure postcapillary venules, and some is removed by bulk flow via the local lymphatic vessels. It is only the remainder that then moves into the low-pressure pleural space.
In situations where systemic pressure varies, the pleural liquid protein concentration varies in concert. For example, systemic hypertensive rats have a lower pleural liquid protein-to-plasma protein concentration ratio than do normotensive rats (0.42 versus 0.55), even though their pulmonary pressures are the same. During development from the fetus to the adult, systemic blood pressure generally rises and pulmonary pressure falls. In a study in sheep, the pleural protein ratio decreased with development, as would be expected if the pleural liquid originated from the high-pressure systemic vessels.

Of the two pleural membranes, the parietal is thought to be more important than the visceral for normal pleural liquid formation. The arguments in favour of this view are as follows:

The parietal pleural microvessels are closer to the pleural space (10 to 12 micrometres) than are those of the visceral pleura (20 to 50 micrometres).

The parietal pleural microvessels probably have a higher filtration pressure than do the visceral bronchial microvessels, which are known to empty into the low-pressure pulmonary veins.

(From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)
The parietal membrane has a consistent anatomy and thickness over its extent in the body and among different species; the visceral membrane varies greatly. Pleural liquid formation rates are similar among species, even when the species have significantly different visceral pleural structures and circulations. Sheep, with a thick pleura and systemic visceral circulation, have similar pleural liquid formation rates as do dogs and rabbits, which have similar parietal pleural anatomy as sheep, but have very different visceral pleural anatomy. If the bronchial circulation of the thick visceral pleura in sheep did contribute, one would expect the liquid formation rate of sheep to be higher than either of the other two species. The formation of pleural liquid is dependent upon a balance of hydrostatic pressures (microvascular minus pleural) opposed by the counterbalancing osmotic pressures (microvascular minus pleural). These pressures can be quantified by application of Starling's equation. A balance of pressures has been proposed that estimates an average 14 cm H2O driving pressure for movement of liquid into the pleural space from the parietal pleura versus 9 cm H2O from the visceral pleura.

Alterations of the balance that could increase pleural liquid formation include: an elevation of systemic microvascular pressure (e.g., from systemic venous hypertension), a decrease in pleural pressure (e.g., in atelectasis), or a decrease in systemic protein concentration (e.g., with hypoproteinaemia). Another possibility, an increase in pleural liquid protein concentration, is probably not relevant clinically. The alteration in balance would presumably be transient and followed by a new balance at a different combination of hydrostatic and countering osmotic pressures.

1.1.3 Pleural liquid absorption

Because the normal situation is a steady state, the absorption rate of pleural liquid should equal the formation rate. If excess liquid is introduced into the pleural space, however, the rate of absorption increases several-fold, from the baseline rate of 0.01 to 0.02 mL/kg per hour to 0.22 to 0.28 mL/kg per hour.

The route of exit of the pleural liquid has been debated, in part because of the difficulty studying the pleural space. Various proposals have included reabsorption by the mesothelial cells themselves and passive flow of pleural liquid into the "low" pressure interstitial tissues of the lung. Nonetheless, current evidence supports the conclusion that the liquid exits the pleural
space via the lymphatic stomata of the parietal pleura. This conclusion is based upon knowledge of the physical forces operating at the pleural tissue and the evidence for bulk flow as opposed to diffusion.

**Physical forces**

Our current understanding of the physical forces operating at the pleural spaces do not support an important role for active transport or uptake by capillaries in the absorption of pleural liquid. The intrapleural pressure is lower than the interstitial pressure of either of the pleural tissues. With this pressure difference, a gradient of pressure directs liquid movement into but not out of the pleural space.

The pleural membranes are leaky, offering little resistance to the movement of liquid and protein, as has been shown for peritoneal mesothelium. Such a condition favours the passive movement of liquid, proteins, and other molecules. This is the underlying characteristic that allows for successful dialysis across the peritoneal membranes.

Mesothelial cells have not been shown to generate an electric potential difference, which would be expected if mesothelial cells moved ions by active transport. Although pleural liquid has been reported to be alkaline with a higher bicarbonate concentration than plasma, there is no evidence yet for mesothelial participation in generating a bicarbonate gradient. Furthermore, it is difficult to explain how the mesothelium could maintain a transport gradient since it is a leaky membrane. Another explanation for ionic differences across a semipermeable membrane is by the passive distribution of ions in response to a difference in protein concentration (a "Donnan equilibrium").

**Evidence for bulk flow**

The majority of liquid appears to exit the pleural space by bulk flow, not by diffusion. At least four findings underlie this assertion.

Pleural liquid protein concentration does not change as a hydrothorax is absorbed. With bulk flow, liquid and protein are removed together, and the protein concentration of the liquid remaining in the pleural space does not change. With diffusion, however, proteins would diffuse at a slower rate than the liquid, resulting in a progressive increase in protein concentration.

The absorption rates of pleural liquid are constant despite differences in protein concentration. If diffusion were predominant, the presence of protein would be expected to slow the removal of the pleural liquid because the higher protein osmotic pressure would reduce the pressure gradient for flow out of the pleural space.
Absorption rates are constant despite changes in pleural liquid volume, at least once the liquid volume rises above some threshold\textsuperscript{26}. If diffusion were the predominant mechanism of absorption, the absorption rate would be expected to change with pleural liquid volume, as the pleural liquid pressure gradient changed.

Erythrocytes are absorbed intact from the pleural space and at nearly the same rate as the liquid and protein. This relatively free exit of erythrocytes from the pleural space indicates that the major route of exit is via holes large enough to accommodate erythrocytes (6 to 8 micrometres for the sheep erythrocytes used in the study). The only possible route then is via the parietal pleural stomata (2 to 10 micrometres) and the lymphatics.

\textbf{Figure 4: Flows of pleural fluid}
A: The sites of preferential pleural fluid filtration through the parietal mesothelium B: The top to bottom and costal to extra-costal intrapleural flow direction C: The sites of preferential removal of pleural fluid into the costal, mediastinal and diaphragmatic pleural lymphatics system.
(From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)

\textbf{1.1.4 Lymphatic flow}
Lymphatic flow is influenced both by intrinsic contractility of the lymph vessels and by extrinsic respiratory movements\textsuperscript{29}. Intrinsic contractility could potentially be altered by hormones, cytokines, or adrenergic stimulation. Respiratory movements may assist lymphatic flow by applying an alternating pressure on the subpleural lymphatics or by expanding and contracting the openings of the lymphatic stomata. Respiratory movements also promote a
continuous intrapleural circulation of pleural liquid, which may favour delivery of pleural liquid to the stomata\textsuperscript{30, 31}.

\textbf{Figure 5: Lymphatic crevices of pleura}
\textit{(Courtesy Boutin C, Astoul Ph)}
1.2 MECHANISMS OF PATHOLOGICAL PLEURAL LIQUID ACCUMULATION

In the normal pleural space, there is a steady state in which there is a roughly equal rate of the formation (entry) and absorption (exit) of liquid.

This balance must be disturbed in order to produce a pleural effusion. Thus, there must be an increase in entry rate and/or a reduction in exit rate. It is likely that both mechanisms contribute to effusion formation for the following reasons:

- An isolated increase in entry rate, unless large and sustained, is unlikely to cause a clinically significant effusion because the absorbing pleural lymphatics have a large reserve capacity to deal with excess pleural liquid. If, for example, artificial hydrothoraxes are instilled into the pleural space of awake sheep, the exit rate can increase to 0.28 mL/kg per hour, which is 28 times the baseline rate\(^{26}\).

- An isolated decrease in exit rate is also unlikely to cause a large effusion because the normal entry rate is low. Even if the exit of liquid ceased entirely, accumulation of liquid would take many days to become evident. As an example, the normal entry rate of 0.01 mL/kg per hour is equivalent to a total of 12 mL/day in a 50 kg woman; at this rate of entry without any exit of liquid, it would take more than one month days for 500 mL to accumulate in the pleural space. Of note, the effusion would presumably be a transudate, since the normal liquid entering the pleural space is low in protein.

1.2.1 Increased fluid entry

Excess liquid filters out of systemic microvessels based on a balance of hydrostatic and osmotic forces across a semipermeable membrane\(^2\). These forces are well described in the Starling equation, in which the hydrostatic forces that force water out of the vessel are balanced by osmotic forces that reabsorb water back into the vessel\(^{32,33}\).

\[
\text{Flow} = k \times \left[ (P_{mv} - P_{pmv}) - s (\pi_{mv} - \pi_{pmv}) \right]
\]

In this equation, \(k\) is liquid conductance of the microvascular barrier, \(P_{mv}\) and \(P_{pmv}\) represent hydrostatic pressure in the microvascular and perimicrovascular compartments, \(s\) is the reflection coefficient for total protein and ranges from 0 (completely permeable) to 1 (completely impermeable), and \(\pi_{mv}\) and \(\pi_{pmv}\) represent protein osmotic pressure in
microvascular and perimicrovascular liquids, respectively. In normal microvessels, there is ongoing filtration of a small amount of low protein liquid. The flow from the microvessels can increase with changes in various parameters of the Starling equation.

**Increase in permeability**

An increase in flow can be due to increases in either liquid conductance (an increase in k) or protein permeability (a decrease in reflection coefficient). If the endothelial barrier becomes more permeable to liquid and protein, for example, there will be an increase in flow of a higher protein liquid. Because absorption does not alter the protein concentration of pleural liquid, pleural liquid with a high protein concentration indicates its origin from a circulation across an area of increased permeability.

![Graph showing lymphatic and extrapleural interstitial pressure](image)

**Figure 6: Lymphatic and extrapleural interstitial pressure**

**A:** Simultaneous recording of tidal volume (top panel), lymphatic ($P_{lymph}$; middle panel, solid line) and extrapleural interstitial ($P_{epl}$; middle panel, dotted line) pressure during spontaneous breathing. The negative sign of the pressure gradient, $\Delta P_{lymph-net} = P_{lymph} - P_{int}$, shown in the bottom panel indicates that, in spontaneous breathing an interstitial to lymphatic fluid flux occurs throughout the whole respiratory cycle.

**B:** Simultaneous recording of $P_{lymph}$, $P_{epl}$ and $\Delta P_{lymph-net}$ during mechanical ventilation in paralysed rats at a breathing frequency of 60 cycles/min (1 Hz). At variance with spontaneous ventilation, both $P_{lymph}$ and $P_{int}$ increase during passive lung inflation. In this conditions, $\Delta P_{lymph-net}$ and thus lymphatic flow is nullified (Modified from Moriondo A et al 2006)

*(From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)*
**Figure 7: Difference of pressures in case of ascites and pleural effusion**

A: the average sub-diaphragmatic abdominal pressure (Pabd, triangles) and Ppl (circles) and trans-diaphragmatic (ΔP, squares, calculated as: ΔP = Pliq - Pabd) pressure in control pre-injection baseline (t < 0) and after dextran injection into the peritoneal (panel A) or pleural (panel B) spaces. The injection was performed at time = 0. A negative ΔP would sustain potential fluid flow from the peritoneal to pleural space. In experimental ascites, the peritoneal to pleural ΔP further increases with respect to the pre-injection baseline value and remain stable for up to 180 min after intraperitoneal injection. Vice versa, pleural effusion reversed the pre injection ΔP value causing the development of a transient pleural to peritoneal ΔP that was completely nullified at 30 min from injection (panel B) (Modified from Moriondo A et al 2007)

* significantly different (p < 0.05) from pre-injection baseline values

(From Negrini D. Physiology and Physiopathology of the pleural space In Thoracoscopy for pulmonologists: A didactic approach. Eds. Ph Astoul, GF Tassi, JM Tschopp. Springer 2014)

**Increase in microvascular pressure**

An elevation in microvascular pressure (Pmv) is usually induced by an elevation in venous pressure. Increases in arterial pressure are less likely to be transmitted to the microvessels because of the high precapillary resistance and autoregulation of arteriolar tone.

Elevations in systemic venous pressure (affecting mostly the parietal pleura) can lead to an increase in pleural liquid formation. Because vascular permeability is unchanged in this setting, the increased flow is associated with a greater sieving of proteins, leading to a filtrate with a lower protein concentration than normal (with a pleural liquid-to-plasma protein ratio of less than 0.15).

Of course, most effusions formed due to increased microvascular pressures, i.e., transudative effusions, have a pleural liquid-to-plasma protein ratio much higher than this, between 0.4 and 0.5. This fact demonstrates that most liquid must arise from a source other than the systemic circulation of the pleural membranes. The likely source is the large non-systemic circulation.
adjacent to the pleural space, namely the pulmonary circulation of the nearby lung. In the normal state, lung interstitial liquid, e.g., lymph, filtered from the low pressure pulmonary circulation has a protein concentration ratio (lung to plasma protein concentration ratio) of 0.7, but with increased flow due to increased pulmonary microvascular pressures, this ratio falls to 0.4-0.5. As shown in studies of anesthetized sheep, this lung interstitial edema liquid is the likely source of the majority of the hydrostatic pleural effusion.

How does the lung liquid reach the pleural space? When the rate of filtrate formation exceeds the absorptive capacity of the lung lymphatics, the filtrate accumulates in the peribronchovascular spaces ("cuffs"). Once in these interstitial spaces, the liquid is not accessible to lung lymphatics. Thus, although the lymphatics are undeniably important in removing liquid as it is filtered from the pulmonary circulation, they cannot account for the clearance of already established edema from the lung. This interstitial edema probably leaves the lung by flowing in response to pressure gradients along the interstitial spaces (interlobular septae, peribronchovascular bundles and visceral pleura) of the lung toward either the mediastinum or the pleural space. The entry of large amounts of lung interstitial liquid into the pleural space will elevate the overall protein concentration of the pleural liquid, giving a ratio of 0.40-0.50, the expected range for a transudative effusion.

Decrease in pleural pressure

A decrease in pleural pressure, as seen with significant atelectasis, may alter the balance of forces described in the Starling equation by reducing the pressures surrounding the nearby microvessels. This decrease in perimicrovascular pressures (Ppmv) can enhance filtration across the microvascular barrier of a low protein liquid (with a pleural liquid-to-plasma protein ratio of less than 0.15). Thus, fluid will accumulate until the pressure balance is restored; the decreased pleural pressure is the likely explanation for the formation of pleural effusions in the setting of atelectasis.

Decrease in plasma osmotic pressure

Hypoproteinemia (due to hypoalbuminemia) will decrease the plasma oncotic pressure (πmv), thereby increasing the forces favouring filtration until the balance is restored. By itself, hypoproteinemia can probably induce small effusions with a low protein concentration. In addition, hypoproteinemia can lower the threshold for effusion formation when other Starling forces are changed. In a study of hospitalized patients with AIDS, for example, hypoproteinemia alone was the apparent cause of 19 percent of all pleural effusions. However, together with other factors, a lower plasma protein concentration may have contributed to effusion formation in many more patients, because, in general, all patients with
effusions had a lower plasma albumin concentration than those without effusion (2.5 versus 3.4 g/dL). In another study, hypoalbuminemia may have enhanced the size of pleural effusions, perhaps by shifting the balance toward increased filtration; in children with parapneumonic pleural effusions, the mean serum albumin concentration was lower in those with large effusions than with small effusions (2.7 versus 3.7 g/dL)\(^4\). Thus, while a low albumin concentration may be an infrequent cause of effusions by itself, it may contribute to effusion formation by other causes.

### 1.2.2 Decreased fluid entry

A decrease in exit rate reflects a reduction in lymphatic function. Because lymphatic function is poorly understood, much of this discussion is speculative. Unlike blood vessels, lymphatic vessels have one-way valves and propel lymph using both their own rhythmic contractions and the respiratory motions of the chest wall. In addition, flow is affected by lymphatic patency, availability of liquid, and the pressures influencing filling (pleural pressure) and emptying (systemic venous pressure) of lymphatics\(^4\)\(^1\)\(^2\).

**Intrinsic factors**

A number of factors can interfere with or inhibit the ability of lymphatics to contract, including:
- Cytokines and products of inflammation (eg, endotoxins)
- Endocrine abnormalities (eg, hypothyroidism)
- Injury due to radiation or drugs (eg, chemotherapeutic agents)
- Infiltration of lymphatics by cancer
- Anatomic abnormalities (eg, yellow nail syndrome)

**Extrinsic factors**

Multiple extrinsic factors can inhibit lymphatic function although the lymphatics themselves are normal. These include:
- Limitation of respiratory motion (eg, diaphragm paralysis, lung collapse, pneumothorax)
- Extrinsic compression of lymphatics (eg, pleural fibrosis, pleural granulomas)
- Blockage of lymphatic stomata (eg, fibrin deposition on pleural surface, pleural malignancy)
- Decreased intrapleural pressure (eg, atelectasis, trapped lung caused by a fibrous rind on the visceral pleura)
- Increased systemic venous pressure - Acutely, increases in venous pressure may decrease lymphatic flow because of the higher downstream pressures; chronically, the lymphatics may be able to adapt.
- Decreased liquid availability - After pneumothorax, for example, liquid will be in contact with fewer lymphatic stomata and may accumulate in the pleural space.

<table>
<thead>
<tr>
<th>Mechanisms</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased pleural fluid formation</td>
<td></td>
</tr>
<tr>
<td>Increased interstitial fluid in the lung</td>
<td>Left ventricular failure, pneumonia, and pulmonary embolus</td>
</tr>
<tr>
<td>Increased intravascular pressure in pleura</td>
<td>Right or left ventricular failure, superior vena cava syndrome</td>
</tr>
<tr>
<td>Increased permeability of the capillaries in the pleura</td>
<td>Pleural inflammation, increased levels of vascular endothelial growth factor</td>
</tr>
<tr>
<td>Increased pleural fluid protein level</td>
<td>Pulmonary edema, hemothorax</td>
</tr>
<tr>
<td>Decreased pleural pressure</td>
<td>Lung atelectasis or increased elastic recoil of the lung, trapped lung</td>
</tr>
<tr>
<td>Increased fluid in peritoneal cavity</td>
<td>Ascites or peritoneal dialysis</td>
</tr>
<tr>
<td>Disruption of the thoracic duct</td>
<td>Chylothorax</td>
</tr>
<tr>
<td>Disruption of blood vessels in the thorax</td>
<td>Hemothorax</td>
</tr>
<tr>
<td>Decreased pleural fluid absorption</td>
<td></td>
</tr>
<tr>
<td>Obstruction of the lymphatics draining the parietal pleura</td>
<td>Cancer, lymphoma</td>
</tr>
<tr>
<td>Elevation of systemic vascular pressures</td>
<td>Superior vena cava syndrome or right ventricular failure</td>
</tr>
<tr>
<td>Disruption of the aquaporin system in the pleura</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Examples of increased and decreased of pleural fluid formation and absorption
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)

1.2.3 Mechanisms of effusion formation

As discussed above, no single mechanism is likely to explain the development of a pleural effusion. It is likely that, for many effusions, multiple factors contribute to effusion formation. In addition, an alteration of one mechanism can lower the threshold for effusion formation later by another mechanism. Thus, a decreased exit rate due to malignant infiltration of lymphatics can gradually reduce the capacity of the lymphatics to drain pleural liquid. An effusion will not form as long as the entry rate remains low; however, if the entry rate should increase, the limitation of the exit rate would reduce the ability of the lymphatics to handle the increased fluid and an effusion could accumulate.

There are few data on the entry and exit rates of pleural liquid in patients with pleural effusions. In two early studies of the turnover of pleural liquid in effusions of patients with a variety of different diseases, the entry rates were highest in tuberculosis but similar among the other disorders. The exit rates were low in malignant and tuberculous effusions compared to the
rates in effusions due to cardiac failure and pulmonary embolism. In one study, for example, calculated lymphatic flow for patients with pulmonary embolism was 0.18 mL/kg per h, which is similar to the maximal exit rate measured for sheep (0.28 mL/kg per h); in comparison, lymphatic flow was lower in the patients with carcinoma or tuberculosis (0.06 and 0.08 mL/kg per h, respectively)\textsuperscript{43}. Interestingly, the entry rate decreased and the exit rate increased after therapy with prednisone in patients with tuberculosis.

Most effusions form due to changes at the level of the pleural membranes, lungs, mediastinum, or diaphragm. On occasion, effusions can develop from abnormalities in other organs such as the central nervous system, kidney, or pancreas.

**Pleura**

Direct involvement of pleural membranes by disease can lead to a pleural effusion by increasing the formation of liquid and interfering with parietal pleural lymphatic function. Hydrostatic pressure elevations can also increase filtration from the pleural membrane microvessels.

**Malignancy**

Malignant pleural effusions (MPE) are probably caused by both mechanisms, increased entry plus decreased exit of fluid. There are patients with rapid entry rates, which can be recognized clinically because the effusion accumulates rapidly after drainage or has a high chest tube drainage rate. In this setting, the tumor has presumably extensively infiltrated pleural capillaries, leading to increased filtration, or is producing cytokines, such as vascular endothelial growth factor (VEGF), that increase capillary permeability\textsuperscript{45, 46}. One study indicated that the mast cell may be the key cell producing cytokines, such as tryptase AB1 and IL1 beta, leading to increased permeability\textsuperscript{47}. Decreased plasma osmotic (?) or oncotic? pressure or decreased pleural pressure could contribute to the enhanced entry of liquid.

On the other hand, malignancy may lead to effusion formation by infiltrating the draining lymphatics or lymph nodes, thereby decreasing the exit rate. In some cases of lymphatic involvement, the decrease in the exit rate appears to be an important mechanism of effusion formation because the effusions can resolve after mediastinal irradiation of involved lymph nodes. In certain MPE, extrapleural involvement of draining lymphatics may be the sole mechanism of effusion formation. Such an isolated exit block may explain the existence of transudative effusions that has been described in approximately 10 percent of patients with MPE\textsuperscript{48}. 
**Tuberculosis**

Tuberculosis involves the pleural membranes with a granulomatous reaction that may lead to both an increased entry rate and decreased exit rate of liquid. The high protein concentration of tuberculous pleural effusions indicates its origin from capillaries involved with an intense inflammation. The exit rate, on the other hand, has been found to be low in both patients and animals with experimental models of BCG pleurisy. The low exit rate may indicate that inflammation of the parietal pleural by tuberculosis is extensive and intense enough to compromise pleural lymphatic function.

For patients with pleural malignancy or tuberculosis, the synergistic combination of increased entry and decreased exit of liquid may explain the massive effusions that can accumulate.

**Pleural microvessel hydrostatic pressure elevation**

An increase in the microvascular pressure of pleural vessels can increase filtration in a variety of ways. Systemic venous pressure elevation both increases filtration from the parietal pleural microvessels and decreases lymphatic drainage into the venous system. In comparison, an elevation in pulmonary venous pressure increases filtration from the visceral pleura.

**Pulmonary embolism**

Pulmonary embolism can increase entry rates of liquid by injuring pulmonary and adjacent pleural systemic circulations, by elevating hydrostatic pressures in pulmonary veins and/or systemic veins, and perhaps by lowering pleural pressure due to atelectasis. Pulmonary embolism may also decrease exit rates of pleural liquid by increasing the systemic venous pressure (thereby hindering lymphatic drainage) or perhaps by decreasing pleural pressure (thereby hindering lymphatic filling). The observation that all effusions due to pulmonary embolism were exudates suggests a key role for vascular injury; however, hydrostatic pressure changes probably also contribute to the formation of the effusions. As in acute lung injury, when microvessels are injured, small changes in hydrostatic pressure can have a large effect on fluid flux.

**Superior vena cava syndrome**

The mechanism of effusion formation in patients with the superior vena cava syndrome has only been studied in a few cases. Apparently, the entry rate of liquid into the pleural space is increased. The most detailed study has been in one patient who was shown to have a transudative effusion with flow through the thoracotomy tube of approximately 500 mL/day. In another study of volume-loaded dogs, an elevation in systemic venous pressure for two hours plus a decrease in plasma osmotic pressure led to a significant increase in pleural liquid entry and formation of an effusion.
The exit rate may also be decreased acutely since the lymphatics must pump against a higher downstream pressure. With chronic pressure elevations, however, the lymphatics may adapt and resume a more normal capacity. In a series of 27 patients with chronic systemic venous pressure elevation due to pulmonary hypertension, for example, no effusions were found on ultrasonography.53

**Brachiocephalic venous obstruction**

This entity has been identified as a cause of persistent, sometimes intractable transudative effusions. In particular, the obstruction, often in a patient undergoing hemodialysis, is thought to increase pleural microvascular hydrostatic pressure, and thereby increase pleural liquid formation and decrease lymphatic clearance.54 The effusion can resolve after angioplasty of the occluded vessel.55

**Lung**

The lung is a potentially large source of liquid immediately adjacent to the pleural space. Lung interstitial liquid can move into the pleural space along a pressure gradient and across leaky pleural membranes.56

- **Acute lung injury**

From animal studies in which injury is limited to the lung, it is clear that large amounts of lung liquid can move into the pleural space. After chemical or hyperoxia-induced lung injury in animals, for example, an increased permeability lung edema developed and was followed by high protein pleural effusions in approximately two hours.57 58 59 With hyperoxic lung injury in rats, the movement of liquid from lung to pleural space could be traced by the movement of a specific marker.60 In sheep given intravenous oleic acid, liquid leaving the lung across the visceral pleura could be collected in a surrounding bag and quantified as representing almost 20 percent of the lung edema.57

These observations suggest that pleural effusions should be common in patients with acute lung injury. In a radiographic study of patients with pulmonary edema, for example, pleural effusions were found to be as common in patients with acute lung injury (36 percent) as in patients with hydrostatic pulmonary edema (40 percent).61 Other lung injuries, such as pneumonia or pulmonary embolism, can also result in effusion formation due to the movement of high protein lung interstitial liquid into the pleural space.

- **Hydrostatic pulmonary edema**

In heart failure, in contrast to lung injury, the abnormalities are not limited to the lung; as a result, identification of the source of the pleural liquid is more difficult. An elevation in systemic venous and pulmonary venous pressures should lead to increased filtration from both
pleural membranes, decreased absorption via pleural lymphatics, and increased filtration into
the lung with movement of lung edema into the pleural space.
Several studies suggest that the contribution of lung edema may be the most important. In an
experimental study in which pressures were elevated in the systemic venous, the pulmonary
venous, or both circulations, the most liquid appeared after systemic pressure elevation52. However, this study lasted only two hours and lung edema takes at least two hours to accumulate and then to flow to the pleural space57 59. It is therefore likely that the contribution from the lung was not yet evident. The observation in a clinical study of patients with heart failure that the presence of pleural effusions by ultrasound correlated better with elevated pulmonary venous pressures than systemic venous pressures is compatible with the importance of a lung contribution62, although a visceral pleural contribution cannot be excluded. A later study of patients with pulmonary hypertension showed that isolated increases in systemic venous pressure, at least when chronic, did not cause edema formation53.
Studies in anesthetized sheep addressed the contribution of the lung (and visceral pleura) more
directly by isolating the lung in an impermeable bag. Lung edema was created by volume
loading to elevate the pulmonary capillary wedge pressure by 10, 20 or 30 cmH2O. Liquid
began to leak from the lung, and by two hours after stable pressure elevation, the lung liquid
flow had reached a steady state. The lung liquid had the same protein concentration as lung
lymph and interstitial liquid later harvested from peribronchovascular spaces ("cuffs"). The
amount of liquid flowing from the lung could account for the pleural effusions found in other
closed-chest volume loaded sheep. The edema that cleared the lung into the pleural space
accounted for almost 23 to 29 percent of all edema formed. Thus, the contribution of the pleural
route to edema clearance appears to be similar for hydrostatic edema and for increased
permeability edema with acute lung injury57. These observations suggest that the pleural route
of edema clearance may be an important additional safety factor protecting against alveolar
flooding.
The protein concentration in the transudative effusions seen with heart failure also argues
strongly that the effusions consist mostly of lung interstitial liquid. As discussed above, liquid
derived from increased filtration across systemic vessels would be expected to have a very low
protein concentration with a pleura-to-plasma protein concentration ratio of less than 0.15.
However, transudative effusions have a higher protein concentration ratio (approximately 0.40
to 0.50), similar to the ratio in pulmonary filtrate (eg, in lung lymph)38.
The protein concentration of pleural fluid and the pleural-serum protein concentration ratio can
increase with acute diuretic therapy, so that a true transudate may yield indices suggestive of
an exudate. Although not all studies have shown a change from transudative to exudative chemistries following diuresis, this phenomenon must be considered when interpreting pleural chemistries in patients following a significant diuresis. 

**Extrapleural or extrapulmonary**

Excess liquid from any tissue in the body may find its way to the pleural space. The excess fluid would be attracted toward the pleural space by the subatmospheric pressure there. Then, once adjacent to the pleural membranes, the excess fluid could move across, either via holes or tears in the mediastinal pleura or diaphragm or via direct flow across the permeable pleural membranes.

- **Mediastinum**

Effusions have been described following mediastinal inflammation due to esophageal variceal sclerotherapy and esophageal perforation. Chyle can also collect in the mediastinum from a break in the thoracic duct and decompresses into the pleural space, producing a chylothorax. Fluid from a pancreatic pseudocyst can also flow via pressure gradients into the mediastinum and then decompress into the pleural space. Usually decompression of the mediastinal collection is achieved when there is flow into one pleural space and thus, these effusions tend to be unilateral.

- **Diaphragm**

Liquid can cross the diaphragm in several ways. Peritoneal liquid can potentially reach the pleural space by diffusion across the two mesothelial layers. The effusions with a high amylase content associated with pancreatitis may form in this way. There may also be transmitted inflammation as, for example, if pancreatitis generates an adjacent pleurisy and increased pleural capillary filtration.

The above mechanisms provide an explanation for slow transfer of liquid across the diaphragm; however, a different mechanism is needed to explain rapid transfer of liquid across the diaphragm. Although some have speculated that liquid might cross the diaphragm via lymphatics, there is no evidence for the existence of direct lymphatic channels connecting the peritoneal and pleural spaces across the diaphragm. In studies in which radiocontrast dye is instilled into the peritoneal space, the dye clearly drains into lymphatics that travel to the mediastinum; no dye enters the pleural space. Therefore, the only likely mechanism for rapid movement of liquid across the diaphragm is through acquired defects in the diaphragm. The defects apparently form at points of weakness in the muscular webbing of the diaphragm. Based on observations during video-assisted thoracoscopy (VATS), the defects have various
morphologies from blebs to fenestrations\textsuperscript{71}. They may be missed, even on direct inspection of the diaphragm, unless pressure is applied to expand them.
1.3 DIAGNOSTIC EVALUATION OF PLEURAL EFFUSIONS

Determining the cause of a pleural effusion is greatly facilitated by analysis of the pleural fluid. Thoracentesis is a simple bedside procedure with imaging guidance that permits fluid to be rapidly sampled, visualized, examined microscopically, and quantified for chemical and cellular content. A systematic approach to analysis of the fluid in conjunction with the clinical presentation should allow the clinician to diagnose the cause of an effusion in about 75 percent of patients at the first clinical evaluation:
- A definitive diagnosis, provided by the finding of malignant cells or specific organisms in the pleural fluid, can be established in approximately 25 percent of patients.
- A presumptive diagnosis, based on the pre-thoracentesis clinical impression, can be substantiated by pleural fluid analysis in an additional 50 percent of patients.

Even with a nondiagnostic thoracentesis, pleural fluid analysis can be useful in excluding other possible causes, such as infection, or guiding subsequent diagnostic studies. Thus, clinical decision-making information can be gained from pleural fluid analysis in over 90 percent of patients.

An approach to pleural fluid analysis will be presented here. Pleural imaging, the technique of thoracentesis, and an approach to pleural effusions of uncertain etiology after the initial evaluation are discussed separately.

1.3.1 Thoracentesis

Indications
The indication for diagnostic thoracentesis is the new finding of a pleural effusion. Observation, in lieu of diagnostic thoracentesis, may be warranted in uncomplicated heart failure and viral pleurisy. In the former setting, the clinical diagnosis is usually secure; in the latter, there is typically a small amount of fluid. However, if the clinical situation is atypical or does not progress as anticipated, thoracentesis should be performed.

Conditions diagnosed by thoracentesis
Only a select number of diagnoses can be established definitively by thoracentesis. These include effusions as a result of malignancy, empyema, tuberculous pleurisy, fungal infection
of the pleural space, chylothorax, cholesterol effusion, urinothorax, esophageal rupture, hemothorax, peritoneal dialysis, and extravascular migration of a central venous catheter. Pleural fluid ANA titres ≥1:160, and a pleural fluid to serum ANA ratio ≥1 had previously been considered diagnostic of lupus pleuritis. However, none of these findings occurs solely in lupus pleuritis. Most clinical laboratories no longer perform lupus cell preparation tests, which are lengthy and complex. Although LE cells may be detected by pleural fluid cytology, this finding has low diagnostic utility. This is because LE cells have been identified in non-lupus related effusions (eg, malignancy, rheumatoid arthritis). Similarly, small case series suggest that pleural fluid ANA titre ≥1:160 is not diagnostic but remains a sensitive (92 to 100 percent) tool for detecting lupus pleuritis in patients with a known diagnosis of lupus. However, ANA titre lacks specificity (83 percent) as it can also be found in exudative, parapneumonic and malignancy-associated effusions. Even an extremely high pleural fluid ANA >1:640 can occur in MPE. Using the ratio of pleural effusion to serum ANA of ≥1 further reduces the sensitivity and the specificity to 75 and 78, respectively. The ANA staining pattern in pleural fluid does not provide any diagnostic value for lupus pleuritic. Thus, measuring ANA has better negative predictive value and appears to be more useful for excluding the diagnosis of lupus pleuritis, particularly in patients who have a known diagnosis of SLE.

### 1.3.2 Pleural fluid analysis

Tests routinely performed on pleural fluid include cell count, pH, protein, lactate dehydrogenase (LDH), and glucose. Additional commonly performed tests in selected patients include amylase, cholesterol, triglycerides, N-terminal pro-BNP, adenosine deaminase, gram and AFB stain, bacterial and AFB culture, and cytology.

Gross appearance

Initial diagnostic clues can be obtained by gross inspection of pleural fluid as it is being aspirated from the patient's chest.

Characterization

The pleural fluid is next characterized as either a transudate or an exudate.

Exudates

In contrast, exudative effusions present more of a diagnostic challenge. Disease in virtually any organ can cause exudative pleural effusions by a variety of mechanisms, including
infection, malignancy, immunologic responses, lymphatic abnormalities, non-infectious inflammation, iatrogenic causes, and movement of fluid from below the diaphragm. Exudates result primarily from pleural and lung inflammation (resulting in increased capillary and pleural membrane permeability) or from impaired lymphatic drainage of the pleural space (resulting in decreased removal of protein and other large molecular weight constituents from the pleural space). Exudates can also result from movement of fluid from the peritoneal space, as seen with acute or chronic pancreatitis, chylous ascites, and peritoneal carcinomatosis.

**Transudates**

Transudates result from imbalances in hydrostatic and oncotic pressures in the chest, as occur with CHF and nephrosis, or conditions external to the pleural space. Examples of the latter include movement of fluid from the peritoneal, cerebrospinal, or retroperitoneal spaces, or from iatrogenic causes, such as crystalloid infusion through a central venous catheter that has migrated into the mediastinum or pleural space. Nevertheless, transudates have a limited number of diagnostic possibilities that can usually be discerned from the patient's clinical presentation.

<table>
<thead>
<tr>
<th>Types</th>
<th>Exudates</th>
<th>Transudates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>Malignancy</td>
<td>Left ventricular failure</td>
</tr>
<tr>
<td></td>
<td>Parapneumonic effusions</td>
<td>Liver cirrhosis</td>
</tr>
<tr>
<td></td>
<td>Tuberculosis</td>
<td></td>
</tr>
<tr>
<td>Less common</td>
<td>Pulmonary embolism</td>
<td>Hypoalbuminemia</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis and other autoimmune pleuritis</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td></td>
<td>Benign asbestos effusion</td>
<td>Hypothyroidism</td>
</tr>
<tr>
<td></td>
<td>Pancreatitis</td>
<td>Nephrotic syndrome</td>
</tr>
<tr>
<td></td>
<td>Post-myocardial infarction</td>
<td>Mitral stenosis</td>
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<td></td>
<td>Post-coronary artery bypass surgery</td>
<td></td>
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<tr>
<td>Rare</td>
<td>Yellow nail syndrome</td>
<td>Constrictive pericarditis</td>
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<tr>
<td></td>
<td>Other lymphatic disorders (lymphangiopleomyomatosis)</td>
<td>Urinothorax</td>
</tr>
<tr>
<td></td>
<td>Drugs</td>
<td>Meigs/syndrome</td>
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<tr>
<td></td>
<td>Fungal infections</td>
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</tr>
</tbody>
</table>

Table 2: Causes of transudative and exudative pleural effusions
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)

**Diagnostic criteria**

The Light's Criteria Rule is a traditional method of differentiating transudates and exudates that measures serum and pleural fluid protein and LDH\textsuperscript{85}. Abbreviated versions of Light's Criteria Rule have similar diagnostic accuracy and have been recommended for clinical use\textsuperscript{86, 87}. 

27
According to the traditional Light's Criteria Rule, if at least one of the following three criteria (i.e., component tests of the rule) is fulfilled, the fluid is defined as an exudate:
- Pleural fluid protein/serum protein ratio greater than 0.5, or
- Pleural fluid LDH/serum LDH ratio greater than 0.6, or
- Pleural fluid LDH greater than two-thirds the upper limits of the laboratory's normal serum LDH.

Combining results of two or more dichotomous tests into a diagnostic rule, as done by the Light’s Criteria Rule, wherein only one component test result needs to be positive to make the rule result positive always increases sensitivity at the expense of decreasing specificity of the rule. As would be expected, therefore, the sensitivity of the Light’s Criteria Rule is higher than the sensitivity of each of the three component tests of the rule but the specificity of the rule is lower than its individual components. This trade-off of higher sensitivity for lower specificity in the design of Light’s Criteria Rule is appropriate for evaluating pleural fluid because it is important that exudates not be missed, since they can have important prognostic implications. Some transudates, however, may be misclassified as an exudate because of the decreased specificity of the rule.

Light's criteria have been criticized for including both the pleural fluid LDH/serum LDH ratio and the pleural fluid LDH because they are highly correlated.

Alternative diagnostic criteria also exist. A meta-analysis of eight studies (1448 patients) examined pleural fluid tests and found that several tests identified exudates with accuracy similar to those used in Light's criteria, but did not require concurrent measurement of serum protein or LDH. Proposed two-criteria and three-criteria diagnostic rules, which require one criterion to be met to define an exudate, include:

**Two-test rule:**
- Pleural fluid cholesterol greater than 45 mg/dL
- Pleural fluid LDH greater than 0.45 times the upper limit of the laboratory's normal serum LDH

**Three-test rule:**
- Pleural fluid protein greater than 2.9 g/dL (29 g/L)
- Pleural fluid cholesterol greater than cholesterol 45 mg/dL (1.165 mmol/L)
- Pleural fluid LDH greater than 0.45 times the upper limit of the laboratory's normal serum LDH

The previous pleural fluid LDH cut-off point for differentiating between exudates and transudates in the traditional Light's criteria rule was 67 percent of (or 0.67 times) the upper
limit of normal serum LDH. This has been changed to 45 percent, based on reanalysis of each criterion individually\textsuperscript{87}. All available tests may misclassify pleural fluid as exudates or transudates when values are near the cut-off points\textsuperscript{87}. Thus, clinical judgment is required when evaluating patients with borderline test results\textsuperscript{85 89}.

Table 3: Light’s criteria for identifying transudates and exudates
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleural fluid protein divided by serum protein</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Pleural fluid lactate dehydrogenase (LDH) divided by serum LDH</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>Pleural fluid LDH&gt;2/3 (67%) the upper normal limit for serum LDH</td>
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</tr>
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</table>

**Chemical analysis**

The measurement of pleural fluid protein and LDH, glucose, pH, cholesterol, triglycerides, and amylase can provide useful information.

**Proteins**

Most transudates have absolute total protein concentrations below 3.0 g/dL (30 g/L), although acute diuresis in heart failure can elevate protein levels into the exudative range\textsuperscript{64 66}. However, such patients have a serum to pleural fluid albumin gradient (the difference between the serum and pleural values) greater than 1.2 g/dL (12 g/L), which correctly categorizes their effusions as transudates\textsuperscript{63 90}. Elevated blood N-terminal pro-brain natriuretic peptide (NT-proBNP) also supports the diagnosis of heart failure when Light’s criteria yield results in the exudative range\textsuperscript{91}.

Tuberculous pleural effusions virtually always have total protein concentrations above 4.0 g/dL (40 g/L)\textsuperscript{85}.

When pleural fluid protein concentrations are in the 7.0 to 8.0 g/dL (70 to 80 g/L) range, Waldenström’s macroglobulinemia and multiple myeloma should be considered\textsuperscript{92 93}.

**LDH**

The level of pleural fluid lactic dehydrogenase (LDH) is one of the key criteria for differentiating transudates and exudates.

Several specific disease associations have been noted with pleural fluid protein and LDH levels:
- Pleural fluid LDH levels above 1000 IU/L (with upper limit of normal for serum of 200 IU/L) are characteristically found in empyema, rheumatoid pleurisy, and pleural paragonimiasis, and are sometimes observed with malignancy\textsuperscript{94 95 96}.

- Pleural fluid secondary to Pneumocystis jirovecii pneumonia has the characteristic finding of a pleural fluid/serum LDH ratio greater than 1.0 and a pleural fluid/serum protein ratio of less than 0.5\textsuperscript{97}. Urinothorax is another cause of elevated pleural fluid LDH associated with low pleural fluid protein levels\textsuperscript{98}.

**Cholesterol**

Pleural cholesterol is thought to be derived from degenerating cells and vascular leakage from increased permeability. Measurement of pleural cholesterol has been used to improve the accuracy of differentiating transudative and exudative effusion. A pleural cholesterol level of greater than 45 mg/dL is not by itself a definitive criterion for an exudate, but does figure in the two and three-test rules as noted above\textsuperscript{87}.

An elevated cholesterol >250 mg/dL defines a cholesterol effusion (also known as pseudochylothorax or chyliform effusion), which occurs in patients with long-term effusions.

**Triglycerides**

Elevated pleural fluid triglyceride concentrations greater than 110 mg/dL supports the diagnosis of a chylothorax, a level less than 50 mg/dL excludes a chylothorax with reasonable likelihood, and an intermediate level between 50 and 110 mg/dL should be followed by lipoprotein analysis of the pleural fluid\textsuperscript{99}.

**Glucose**

A low pleural fluid glucose concentration (less than 60 mg/dL [3.33 mmol/litre], or a pleural fluid/serum glucose ratio less than 0.5) narrows the differential diagnosis of the exudate to the following possibilities\textsuperscript{100}:

- Rheumatoid pleurisy
- Complicated parapneumonic effusion or empyema
- Malignant effusion
- Tuberculous pleurisy
- Lupus pleuritis
- Esophageal rupture.

All transudates and all other exudates have pleural fluid glucose concentration similar to that of blood glucose.

The mechanism responsible for a low pleural fluid glucose depends upon the underlying disease. Specific examples include:
- Decreased transport of glucose from blood to pleural fluid with rheumatoid pleurisy or malignancy.  
- Increased utilization of glucose by constituents of pleural fluid, such as neutrophils, bacteria (empyema), and malignant cells.

The lowest glucose concentrations are found in rheumatoid pleurisy and empyema, with glucose being undetectable in some cases. In comparison, when the glucose concentration is low in tuberculous pleurisy, lupus pleuritis, and malignancy, it usually falls into the range of 30 to 50 mg/dL (1.66 to 2.78 mmol/litre).

**pH**

Pleural fluid pH should always be measured in a blood gas machine rather than with a pH meter or pH indicator paper, as the latter will result in inaccurate measurements. A pleural fluid pH below 7.30 with a normal arterial blood pH is found with the same diagnoses associated with low pleural fluid glucose concentrations. The pH of normal pleural fluid is approximately 7.60, due to a bicarbonate gradient between pleural fluid and blood. Thus, a pH below 7.30 represents a substantial accumulation of hydrogen ions. Transudates generally have a pleural fluid pH in the 7.40 to 7.55 range, while the majority of exudates range from 7.30 to 7.45.

The mechanisms responsible for pleural fluid acidosis (pH < 7.30) include:
- Increased acid production by pleural fluid cells and bacteria (empyema).
- Decreased hydrogen ion efflux from the pleural space, due to pleuritis, tumor, or pleural fibrosis. Specific examples include malignancy, rheumatoid pleurisy and tuberculous pleurisy.

A low pleural fluid pH has diagnostic, prognostic, and therapeutic implications for patients with parapneumonic and MPE. Patients with a low pleural fluid pH MPE have a high initial positive yield on pleural fluid cytology. They also tend to have a shorter survival and poorer response to chemical pleurodesis than those with a pH > 7.30, although the strength of these associations do not provide prognostic value for individual patients. Clinicians should not use the pleural fluid pH as the sole criterion for the decision to recommend pleurodesis.

A parapneumonic effusion with a low pleural fluid pH (≤ 7.15) indicates a high likelihood of necessity for pleural space drainage.

**Amylase** — Although not routinely tested in pleural fluid samples, amylase measurements can assist in settings wherein pancreatic or esophageal etiologies of an effusion appear possible. The finding of an amylase-rich pleural effusion, defined as either a pleural fluid amylase greater than the upper limits of normal for serum amylase or a pleural fluid to serum amylase ratio...
greater than 1.0, narrows the differential diagnosis of an exudative effusion to the following major possibilities:
- Acute pancreatitis
- Chronic pancreatic pleural effusion
- Esophageal rupture
- Malignancy.
Pleural fluid amylase, however, has low discriminative value for differentiating benign from MPE so it is not routinely performed for this reason.

Other rare causes of an amylase-rich pleural effusion include pneumonia, ruptured ectopic pregnancy, hydronephrosis, and cirrhosis. Pancreatic disease is associated with pancreatic isoenzymes, while malignancy and esophageal rupture are characterized by a predominance of salivary isoenzymes.

Adenosine deaminase
Measurement of adenosine deaminase (ADA) may be helpful with a differential diagnosis of malignant versus tuberculous pleurisy when an exudative effusion is lymphocytic, but initial cytology and smear and culture for tuberculosis are negative. The level of ADA is typically greater than 35 to 50 U/L in tuberculous pleural effusions. Specificity is increased when the lymphocyte to neutrophil ratio is greater than 0.75 and the ADA is greater than 50 U/L. False negatives and positive ADA results do occur, so ADA results need to be considered in the context of other features of the patient’s clinical presentation. ADA testing may be more valuable for ruling in the diagnosis of tuberculous pleurisy in geographic locations with high prevalence of tuberculosis, although the negative predictive value of ADA testing remains high in lower tuberculosis prevalence regions. Some studies suggest greater diagnostic value of pleural fluid interferon gamma in differentiating malignant from tuberculous lymphocytic exudates, but the lower cost and ready availability has promoted the use of ADA testing in this setting.

N-terminal pro-BNP
Several studies have demonstrated that N-terminal pro-brain natriuretic peptide (NT-proBNP) is elevated in the pleural fluid of patients who have heart failure and a pleural effusion. Measuring pleural fluid NT-proBNP, however, has no added value as compared with blood NT-proBNP levels, which are elevated in heart failure and have a high degree of correlation with pleural NT-proBNP results. Blood NT-proBNP testing is useful for diagnosing a cardiogenic pleural effusion in patients whose pleural fluid appears exudative (eg, due to diuresis).
Tumor markers
No single pleural fluid tumor marker is accurate enough for routine use in the diagnostic evaluation of pleural effusion\textsuperscript{131}. Measuring a panel of tumor markers (eg, carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 125, CA 15-3, CA 19-9, cytokeratin fragment CYFRA 21-1) in pleural fluid has also been examined\textsuperscript{132, 133}. At levels that provide 100 percent specificity, individual sensitivities are less than 30 percent and a combination of four markers (CEA, CA 125, CA 15-3, and CYFRA 21) only reached 54 percent sensitivity in one study\textsuperscript{133}. Similar results were reported in a meta-analysis that looked at various combinations\textsuperscript{132}.
Mesothelin is a glycoprotein that is highly over expressed in malignant mesothelioma cells\textsuperscript{134}. Elevated levels of soluble mesothelin-related peptides (SMRPs), cleaved or unbound peptide fragments of mesothelin, have been found in pleural fluid and/or serum of patients with mesothelioma, ovarian, and pancreatic cancer. The role of SMRPs in the diagnosis of pleural mesothelioma is discussed separately.

Nucleated cells
The total pleural fluid nucleated cell count is virtually never diagnostic. There are, however, some settings in which the count may be helpful:
- Counts above 50,000/microL are usually found only in complicated parapneumonic effusions, including empyema.
- Exudative effusions from bacterial pneumonia, acute pancreatitis, and lupus pleuritis usually have total nucleated cell counts above 10,000/microL\textsuperscript{135}.
- Chronic exudates, typified by tuberculous pleurisy and malignancy, typically have nucleated cell counts below 5000/microL\textsuperscript{135}.

The timing of thoracentesis in relation to the acute pleural injury determines the predominant cell type. The early cellular response to pleural injury is neutrophilic. As the time from the acute insult lengthens, the effusion develops a mononuclear predominance if the pleural injury is not ongoing.

Lymphocytosis
Pleural fluid lymphocytosis, particularly with lymphocyte counts representing 85 to 95 percent of the total nucleated cells, suggests tuberculous pleurisy, lymphoma, sarcoidosis, chronic rheumatoid pleurisy, yellow nail syndrome, or chylothorax\textsuperscript{74, 136}. Carcinomatous pleural effusions will be lymphocyte-predominant in over one-half of cases; however, the percentage of lymphocytes is usually between 50 and 70 percent\textsuperscript{136}. 

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**Eosinophilia**

Pleural fluid eosinophilia (defined by pleural fluid eosinophils representing more than 10 percent of the total nucleated cells) usually suggests a benign, self-limited disease, and is commonly associated with air or blood in the pleural space. However, two studies have noted that malignancy is as prevalent in eosinophilic as noneosinophilic pleural effusions. The differential diagnosis of pleural fluid eosinophilia includes:

- Pneumothorax
- Hemothorax
- Pulmonary infarction
- Benign asbestos pleural effusion
- Parasitic disease
- Fungal infection (coccidioidomycosis, cryptococcosis, histoplasmosis)
- Drugs
- Malignancy (carcinoma, lymphoma)
- Catamenial pneumothorax with pleural effusion

Pleural fluid eosinophilia appears to be rare with tuberculous pleurisy on the initial thoracentesis\textsuperscript{138}.

**Mesothelial cells**

Mesothelial cells are found in small numbers in normal pleural fluid, are prominent in transudative pleural effusions, and are variable in exudative effusions. The major clinical significance of mesothelial cells in exudates is that tuberculosis is unlikely if there are more than 5 percent mesothelial cells\textsuperscript{136} 137 138 142 143.

The evaluation of pleural effusions of undetermined etiology after the above evaluation is discussed separately.

It is important to underline that these specific analysis and the tumoral markers are very rarely performed. Indeed, the routine tests are reported in the table below.

<table>
<thead>
<tr>
<th>Test</th>
<th>Test value</th>
<th>Suggested diagnosis</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine deaminase (ADA)</td>
<td>&gt;40 U/L (667 nkat/L)</td>
<td>Tuberculosis (\textsuperscript{-90%}), empyema (60%), complicated parapneumonic effusion (30%), malignancy (5%), rheumatoid arthritis</td>
<td>In the United States, ADA is no routinely requested because of the low prevalence of tuberculosis pleurisy.</td>
</tr>
<tr>
<td>Cytology</td>
<td>Present</td>
<td>Malignancy</td>
<td>Actively dividing mesothelial cells can mimic and adenocarcinoma.</td>
</tr>
<tr>
<td>Glucose</td>
<td>&lt;60 mg/dL (3.3 mmol/L)</td>
<td>Complicated parapneumonic effusion or empyema, tuberculosis (20%), malignancy (&lt;10%), rheumatoid arthritis</td>
<td>In general, pleural fluids with a low glucose level also have low pH and high LDH levels.</td>
</tr>
<tr>
<td>Lactate dehydrogenase (LDH)</td>
<td>Two thirds of normal for serum LDH</td>
<td>Any condition causing an exudate</td>
<td>Very high levels of pleural fluid LDH (&gt;1,000 U/L) typically are found in patients with complicated parapneumonic pleural effusion and in about 40% of those with tuberculosis pleurisy.</td>
</tr>
<tr>
<td>LDH fluid to serum ratio</td>
<td>&gt;0.6</td>
<td>Any condition causing an exudate</td>
<td>Most patients who meet the criteria for an exudative effusion with LDH but no with protein levels have either parapneumonic effusions or malignancy.</td>
</tr>
<tr>
<td>Protein fluid to serum ratio</td>
<td>&gt;0.5</td>
<td>Any condition causing an exudate</td>
<td>A pleural fluid protein level &gt;3 mg/dL suggests an exudate, but when taken alone this parameter misclassifies more than 10% of exudates and 15% of transudates.</td>
</tr>
<tr>
<td>Red blood cell count</td>
<td>&gt;100,000/mm\textsuperscript{3} (100-10\textsuperscript{5}/L)</td>
<td>Malignancy, trauma, parapneumonic effusion, pulmonary embolism</td>
<td>A fluid hematocrit &lt;1% is nonsignificant.</td>
</tr>
<tr>
<td>White blood cell count and differential</td>
<td>&gt;10.000/mm\textsuperscript{3} (10-10\textsuperscript{5}/L)</td>
<td>Emphyema, other exudates (uncommon)</td>
<td>In purulent fluids, leukocyte count is commonly much lower than expected because dead cells or other debris account for much of the turbidity.</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>&gt;10%</td>
<td>Not diagnostic</td>
<td>The presence of air or blood in the pleural space is a common cause. No diagnosis is ever obtained in an many as third of patients with eosinophilic pleural effusion.</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>&gt;50%</td>
<td>Malignancy, tuberculosis, pulmonary embolism, coronary artery bypass surgery</td>
<td>Pleural fluid lymphocytosis &gt;50% suggests tuberculosis or lymphoma.</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>&gt;50%</td>
<td>Parapneumonic effusion, pulmonary embolism, abdominal diseases</td>
<td>In about 7% of acute tuberculosis pleurisy and 20% of malignant pleural effusions, a neutrophilic fluid predominance can be seen.</td>
</tr>
</tbody>
</table>
Table 4: Routine pleural fluid tests for pleural effusions
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)

<table>
<thead>
<tr>
<th>Test</th>
<th>Test value</th>
<th>Suggested diagnosis</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase</td>
<td>&gt;Upper limit of normal</td>
<td>Malignancy (&lt;20%), pancreatic disease: esophageal rupture</td>
<td>Obtain when esophageal rupture or pancreatic disease is suspected. The amylase in malignancy and esophageal rupture is of the salivary type.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&gt;45 to 60 mg/dL (1.16–1.55 mmol/L)</td>
<td>Any condition causing an exudate</td>
<td>Measure if chylothorax or pseudochylothorax is suspected. This parameter taken alone misclassifies 10% of exudates and 20% of transudates.</td>
</tr>
<tr>
<td>Culture</td>
<td>Positive</td>
<td>Infection</td>
<td>Obtain in all parapneumonic pleural effusions because a positive Gram stain or culture should lead to prompt chest tube drainage.</td>
</tr>
<tr>
<td>Hematocrit fluid to blood ratio</td>
<td>≥0.5</td>
<td>Hemorrhax</td>
<td>Obtain when pleural fluid is bloody. Hemorrhax most often originates from blunt or penetrating chest trauma.</td>
</tr>
<tr>
<td>Interferon</td>
<td>Different cutoff points</td>
<td>Tuberculosis</td>
<td>Consider when ADA is unavailable or nondiagnostic and tuberculosis is suspected.</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>&gt;1,500 pg/mL</td>
<td>Heart failure</td>
<td>If available, consider testing when heart failure is suspected and exudate criteria are met.</td>
</tr>
<tr>
<td>pH I</td>
<td>&lt;7.20</td>
<td>Complicated parapneumonic effusion or empyema, malignancy (&lt;10%), tuberculosis (&lt;10%), esophageal rupture</td>
<td>Obtain in all nonpurulent effusions if infection is suspected. A low pleural fluid pH indicates the need for tube drainage only for parapneumonic pleural effusions.</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>Positive</td>
<td>Infection</td>
<td>Consider when infection is suspected. Sensitivity of polymerase chain reaction to detect Mycobacterium tuberculosis in pleural fluid varies from 40% to 80% and is lower in patients with negative mycobacterial cultures.</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>&gt;110 mg/dL (1.24 mmol/L)</td>
<td>Chylothorax</td>
<td>Obtain when pleural fluid is cloudy or milky. Chylothorax is caused by lymphoma or trauma. Not all chylous pleural effusions appear milky white or whitish.</td>
</tr>
<tr>
<td>Tumor markers</td>
<td>Different cutoff points</td>
<td>Malignancy</td>
<td>Consider when malignancy is suspected and thoracoscopy is being considered. Except for telomerase activity, individual tests tend to have low sensitivity (&lt;30%) when looking for the utmost specificity.</td>
</tr>
</tbody>
</table>

Table 5: Optional pleural fluid tests for pleural effusions
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)

1.3.3 Additional tests for undetermined etiology

Pleural effusions can develop as a result of over 50 different pleuropulmonary or systemic disorders. Following diagnostic thoracentesis, the cause of a pleural effusion is not evident in up to 25 percent of patients. However, no universally accepted definition exists for an "undiagnosed effusion."

This topic will review the approach to pleural effusions for which the diagnosis is unclear after initial clinical assessment and investigation. The initial assessment of a patient with a pleural effusion is presented separately.
Clinical history

The first step for the clinician is to revisit the patient's history, paying particular attention to drugs, occupational exposures, risk factors for pulmonary embolism or tuberculosis, and comorbid conditions.

A careful drug history may reveal that the patient is taking nitrofurantoin, amiodarone, ovarian stimulation therapy, or a drug that can produce a lupus-like syndrome. Occupational asbestos exposure, which might suggest a benign asbestos pleural effusion, may have occurred many years earlier. Benign asbestos effusions are usually unilateral, exudative, and about a third have an elevated pleural eosinophil count.

Many systemic disorders (eg, lupus, hypothyroidism, amyloid, yellow nail syndrome) can cause an effusion and must not be overlooked. Patients with yellow nail syndrome typically have friable, yellow nails that grow slowly, and may also have lymphedema. Abnormal nail fold capillaroscopy has also been reported.

Pleural fluid may originate from extrapleural sources, most commonly from transdiaphragmatic movement. A detailed history and examination for ascites, urinary tract obstruction, and hepatic and pancreatic diseases are important.

Some effusions resolve spontaneously, but the time required for resolution varies depending upon the underlying etiology. Uncomplicated parapneumonic effusions and effusions from pulmonary embolism, tuberculous pleurisy, and postcardiac injury syndrome may persist for several weeks.

Benign asbestos pleural effusion, rheumatoid pleurisy, and radiation pleuritis often persist for months to years. Other effusions that may persist for years include those caused by lymphatic abnormalities (eg, yellow-nail syndrome and pulmonary lymphangiectasia) and trapped lung. MPE, on the other hand, seldom resolve spontaneously.

The diagnosis of trapped lung should be suspected in any patient with a stable chronic pleural effusion, particularly if there is a past history of pneumonia, pneumothorax, thoracic surgery, or hemothorax. A trapped lung occurs when a remote inflammatory process causes a fibrin peel to form on the visceral pleural surface, thereby preventing the lung from expanding to the chest wall. This generates a negative pleural pressure favouring movement of fluid from the parietal pleural capillaries into the pleural space, until a new steady state is reached. Other causes of trapped lung include rheumatoid pleurisy, uremic pleurisy, tuberculosis, and malignancy.
Tuberculous pleurisy affects all age groups and should not be overlooked\textsuperscript{152}. An initial negative skin test does not exclude the diagnosis of pleural tuberculosis; the need for further diagnostic procedures in this setting is dictated largely by the likelihood of tuberculosis exposure. Elevated levels of pleural fluid adenosine deaminase and interferon-gamma increase the likelihood that a lymphocytic effusion is due to tuberculosis\textsuperscript{153}. Empiric treatment for tuberculosis may be considered in selected PPD (purified protein derivative) positive patients with an undiagnosed, lymphocyte-predominant exudate and no alternative likely etiology.

<table>
<thead>
<tr>
<th>Findings</th>
<th>Potential causes of the pleural effusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>History</td>
<td></td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td>Postabdominal surgery, subphrenic abscess, pulmonary embolism</td>
</tr>
<tr>
<td>Asbestos exposure</td>
<td>Mesothelioma, benign asbestos pleural effusion</td>
</tr>
<tr>
<td>Cancer</td>
<td>Malignancy, paramalignant effusions (pulmonary embolism, atelectasis, postobstructive pneumonitis, postradiation therapy)</td>
</tr>
<tr>
<td>Cardiac procedures or myocardial infarction</td>
<td>Pleural effusion secondary to coronary artery bypass surgery, post-cardiac injury syndrome</td>
</tr>
<tr>
<td>Carhosis</td>
<td>Hepatic hydrothorax, spontaneous bacterial pleuritis</td>
</tr>
<tr>
<td>Collapse therapy for pulmonary TB</td>
<td>Tuberculous or pyogenic empyema, pyothorax-associated lymphoma, trapped lung</td>
</tr>
<tr>
<td>Dialysis</td>
<td>Heart failure, uremic pleuritis, pleural effusion secondary to peritoneal dialysis</td>
</tr>
<tr>
<td>Drug use</td>
<td>Drug-related effusions (e.g., desazirh, gonadotrophins, amiodarone)</td>
</tr>
<tr>
<td>Esophageal surgery, dilation or endoscopy</td>
<td>Chylothorax, esophageal perforation</td>
</tr>
<tr>
<td>Heart failure</td>
<td>Heart failure-related effusion</td>
</tr>
<tr>
<td>HIV</td>
<td>Pneumonia, TB, primary effusion lymphoma, Kaposi sarcoma</td>
</tr>
<tr>
<td>Neurosurgery</td>
<td>Intrathoracic migration of ventriculoperitoneal shunt, ventriculothoracic shunt, dural pleural fistula</td>
</tr>
<tr>
<td>Pancreatic disease</td>
<td>Pancreatic effusion (pancreatic-pleural fistula)</td>
</tr>
<tr>
<td>Radiofrequency ablation of lung or liver tumors</td>
<td>Pleuritis secondary to radiofrequency ablation</td>
</tr>
<tr>
<td>Recurrent episodes of pleuritic pain (plus fever, abdominal pain or arthritis)</td>
<td>Familial Mediterranean fever</td>
</tr>
<tr>
<td>Rheumatic autoimmune diseases</td>
<td>Rheumatoid pleurisy, lupus pleuritis, parapneumonic effusion, pulmonary arterial hypertension</td>
</tr>
<tr>
<td>Trauma</td>
<td>Hemorrhage, chylothorax</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>Parapneumonic effusion/empyema, TB, viral pleuritis, lupus pleuritis</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>Lung cancer, TB, pulmonary embolism, parapneumonic effusion</td>
</tr>
<tr>
<td>Weight loss</td>
<td>Cancer, empyema, TB</td>
</tr>
<tr>
<td>Signs</td>
<td></td>
</tr>
<tr>
<td>Ascites</td>
<td>Hepatic hydrothorax, ovarian cancer, Maeg’s syndrome, constrictive pericarditis</td>
</tr>
<tr>
<td>Distended abdominal veins, encephalopathy, spider nevi</td>
<td>Cirrhosis</td>
</tr>
<tr>
<td>Pericardial rub</td>
<td>Acute pericarditis</td>
</tr>
<tr>
<td>S+ gallop, elevated neck veins, positive abdomino-jugular test, displaced apical impulse</td>
<td>Heart failure</td>
</tr>
<tr>
<td>Unilateral calf pain or swelling</td>
<td>Pulmonary embolism</td>
</tr>
<tr>
<td>Yellow dystrophic nails, lymphedema</td>
<td>Yellow nail syndrome</td>
</tr>
</tbody>
</table>

Table 6: Medical history and physical examinations of pleural effusions
(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)
Reanalysis of pleural fluid

The pleural fluid should be analysed carefully: vital diagnostic clues may lie in simple inspection (especially for chyloous effusions) or the smell (in urinothorax), but are often overlooked. Most undiagnosed pleural effusions are exudative, because the differential diagnosis of a transudative effusion is limited and the cause can usually be recognized by the clinical presentation. A few points are worth noting when categorizing an undiagnosed effusion as a transudate or exudate.

One common problem is that transudative pleural fluid from patients receiving diuretic therapy may have an elevated protein level and be mistakenly classified as an exudate. In such cases, the N-terminal pro-brain natriuretic peptide (NTproBNP) level in the pleural fluid, especially if higher than 1500 pg/mL, can be used to determine if the pleural fluid is a transudate due to heart failure \textsuperscript{130, 129}. NTproBNP has been shown to correctly diagnose heart failure as a cause of most effusions that have been misclassified as exudates by Light’s criteria and can help avoid repeated invasive investigations in patients with a strong clinical suspicion of cardiac failure \textsuperscript{154}. If NTproBNP is unavailable, the fluid can be considered a transudate if the total protein gradient (serum minus pleural fluid) is greater than 3.1 g/dL in a patient with convincing clinical picture of heart failure who is taking diuretics \textsuperscript{63}.

Constrictive pericarditis, which can be a sequel of prior cardiac surgery, infection, or remote radiation therapy, can also be difficult to diagnose. The resulting pleural effusion is typically transudative, but can be exudative with effusive-constrictive pericarditis.

Effusions that are classically exudative can be transudative in some cases (eg, malignancy), usually a result of concomitant causes of transudative fluid formation \textsuperscript{155}. One study of 638 MPE from 103 patients reported a significant reduction in pleural fluid protein (8 g/L/100 days) and pH (0.04/100 days) accompanied by a small rise in LDH over time \textsuperscript{156}.

In patients with a lymphocytic effusion, further investigation should be considered for tuberculosis, sarcoidosis, lymphoma, chylothorax, and pseudochoylothorax \textsuperscript{157}. Flow cytometry may be helpful in the evaluation of pleural lymphoma \textsuperscript{158}. Pleural fluid adenosine deaminase may be helpful when tuberculosis is suspected \textsuperscript{159}. A meta-analysis of 63 studies showed that pleural fluid adenosine deaminase (ADA) level provides 92 percent sensitivity and 90 percent specificity in diagnosing tuberculous pleuritic \textsuperscript{160}.

A viral etiology has been postulated for effusions in which no causes were found despite investigation, especially if the effusion resolved without specific treatment. A wide range of viruses have been associated with a pleural effusion \textsuperscript{161}. However, there are no pathognomonic
features that allow easy diagnosis of a viral pleural effusion. No studies to date have studied how common viral infections are in otherwise undiagnosed effusions. Polyserositis involving the pleura and pericardium (and at times the peritoneum) have been associated with autoimmune diseases, especially lupus, though in many cases the cause was not identified. Genetic testing is helpful if familial Mediterranean fever is suspected.

**Follow-up**

The cause of the pleural effusion may become obvious with long-term follow-up, though most cases remain of unknown etiology\textsuperscript{162,163,164}. One study monitored 143 patients who had a non-diagnostic pleural fluid analysis and pleural biopsy. Follow-up ranging from one to six years revealed that 29 (20 percent) had malignancy, and one had tuberculosis\textsuperscript{162}. However, another study found neither malignancy nor tuberculosis during a mean follow-up of 33 months in 53 patients with nonspecific pleuritis\textsuperscript{164}. In a retrospective study of 83 patients with undiagnosed "idiopathic" effusions, 47 resolved, 20 improved, and 16 persisted during follow-up\textsuperscript{165}. Biochemical pleural fluid analysis did not predict these outcomes\textsuperscript{164}. During follow-up, particular attention is paid to identifying "treatable" causes of a pleural effusion (eg, tuberculosis [TB], lymphoma)\textsuperscript{165}. Overall, patients with idiopathic pleural effusions generally have favourable outcomes\textsuperscript{163}. The optimal duration of follow-up has not been defined.

**1.3.4 Other diagnostic techniques**

If clinical examination and pleural fluid analysis fail to result in a diagnosis, additional investigations with imaging and pleural biopsy will be needed. Bronchoscopy is rarely helpful in identifying the cause of an undiagnosed pleural effusion, unless the chest radiograph or computed tomography (CT) show parenchymal abnormalities or the patient presents with hemoptysis\textsuperscript{166,167}.

**Pleural pressure**

Trapped lung can be diagnosed radiologically, or in the appropriate clinical setting, when the decrease in pleural pressure is more than 15 cm H2O per litre of fluid removed. The initial pleural pressure with a trapped lung is typically negative, but a slight positive pressure can also be observed\textsuperscript{151}.
Imaging

CT imaging of the thorax with pleural phase contrast enhancement should be performed in virtually all patients with an undiagnosed pleural effusion. Irregular or thickened pleura with contrast enhancement suggests pleural inflammation or malignancy and identifies optimal sites for CT-guided needle aspiration or cutting needle biopsy. CT may also reveal signs of invasion of underlying or adjacent structures that would suggest MPE. CT pulmonary angiography may be considered if pulmonary embolism (PE) is a consideration. Two studies of 60 and 230 patients with pulmonary emboli showed that 47 to 48 percent had an effusion, the majority of which were small and did not require drainage. Large effusions should alert clinicians to alternative or additional causes of fluid formation other than pulmonary embolism.

Positron emission tomography (PET)/CT has an emerging role: 18-fluorodeoxyglucose (FDG)-avidity confirms, but cannot differentiate between inflammatory and malignant disease. Focal increased uptake of FDG in the pleura and the presence of solid pleural abnormalities on CT are suggestive of MPE, and can guide biopsies. A PET/CT pattern composed of pleural uptake and increased effusion activity had an accuracy of 90 percent in predicting MPE in 31 patients with known extrapulmonary malignancy and a pleural effusion. A negative PET/CT does not exclude malignancy but would favour a benign etiology. PET/CT may also highlight extrapleural abnormalities that may be the cause of the effusion.

Chest ultrasound

Portable ultrasound devices are used also at the bedside to evaluate pleural abnormalities and to guide thoracentesis and related procedures, such as pleural drainage catheter placement and needle aspiration biopsy of pleural or subpleural lung masses. The goals are to improve accuracy and safety in the characterization of pleural disease and performance of pleural access procedures. Thoracic ultrasound has several advantages over traditional radiographic imaging of the pleura, including absence of radiation, better portability, real-time imaging, and the ability to perform dynamic imaging. Ultrasound is substantially better at determining the location of pleural fluid than bedside physical examination and, in experienced hands, is associated with a lower rate of complications during thoracentesis. In addition, ultrasound guidance increases the likelihood of a successful tap compared to using physical examination for guidance. Ultrasound examination of the pleura is more sensitive than a plain chest radiograph at detecting the presence of pleural fluid and differentiating pleural fluid from lung consolidation. Ultrasound guidance is associated with a reduced risk of pneumothorax during thoracentesis.
Thoracic ultrasound has also some disadvantages. It is an operator dependent technology, thus focused, supervised training is needed. Ultrasound is not as good as CT imaging for evaluation of the underlying lung parenchyma in the setting of complex pleural and lung parenchymal disease, and for complicated interventional procedures, such as empyema drainage with a pigtail catheter or biopsy of pleural masses.

These are the indications of chest ultrasound: detection of pleural fluid and pneumothorax also at bedside, guidance for diagnostic and therapeutic thoracentesis and for placement of thoracostomy tubes.

**Pleural biopsy**

Pleural biopsy typically follows CT scan in undiagnosed pleural effusions. A number of techniques for pleural biopsy are available.

Percutaneous techniques include closed pleural biopsy and CT-guided cutting needle biopsy. The former is useful primarily when diseases such as tuberculosis (TB) are suspected (eg, because of a lymphocytic effusion or risk factors for TB). Ultrasound guidance can improve the yield of percutaneous biopsy. CT guided biopsy is useful when a pleural-based mass is visible.

Thoracoscopic pleural biopsy is increasingly used to diagnose malignancy when an obvious mass is not visible on CT, when percutaneous biopsy is negative, or when patchy disease is suspected.

Open pleural biopsy by thoracotomy has been used in the past, but has been largely replaced by thoracoscopic pleural biopsy.

**Closed pleural biopsy**

Closed pleural biopsy is useful in conditions where the pleura is diffusely involved (eg, tuberculous pleuritis or non-caseating granulomata in rheumatoid pleuritis), but less so if involvement is patchy (eg, pleural malignancies). It can be considered in the setting of an undiagnosed lymphocytic effusion, particularly if the percentage of lymphocytes is greater than 80 percent.

Closed pleural biopsy of the parietal pleura with a Cope or Abrams needle is operator dependent. In a retrospective analysis of 75 patients who underwent percutaneous pleural biopsy, sufficient pleural tissue was obtained from 72 percent of patients when three samples were obtained, which increased to 80 percent with four to six samples. For all diagnoses, the sensitivity, specificity, positive predictive value, and negative predictive values were 38, 100, 100, and 51 percent, respectively. Pneumothorax complicated 11 percent of procedures.
The initial pleural biopsy in tuberculous pleuritis is positive in approximately 70 percent of cases. A greater number of biopsies taken at a single session (six or more), or multiple, separate biopsy procedures can increase the sensitivity to 80 percent 184. The combination of pleural fluid adenosine deaminase level and closed pleural biopsy may be adequate to establish a diagnosis of pleural tuberculosis 185.

**CT-guided cutting needle biopsy**

CT-guided cutting needle biopsy is typically used to diagnose malignancy when pleural-based soft tissue masses are identified by CT scan. In a randomized trial, CT-guided biopsy had a sensitivity of 87 percent (versus 47 percent with Abrams needle biopsy) in patients with suspected MPE with negative cytology 180. In another study of 85 cases, the sensitivity and specificity were 76 and 100 percent, respectively, for image guided biopsy for pleural malignancies. A pneumothorax occurred in only 4 percent of cases 186.

**Thoracoscopic pleural biopsy**

Thoracoscopic pleural biopsy, in the form of video-assisted thoracoscopic surgery (VATS) or pleuroscopy (also called medical thoracoscopy) has a diagnostic yield for MPE that is superior to percutaneous pleural biopsy 185 187 188 189 190.

It should be noted that a negative thoracoscopic biopsy does not fully exclude malignant pleural disease. False negative results (around 8 percent) can occur, especially with mesothelioma 191 192 193.

![Image](Pleural lympangitis, Pleural plaques, Anthracotic deposits)

**Figure 9: Example of thoracoscopic images**

*(Courtesy Boutin C, Astoul Ph)*
1.4 MANAGEMENT OF MALIGNANT PLEURAL EFFUSIONS

1.4.1 Introduction

Up to 50 percent of patients with a variety of metastatic malignancies develop a paramalignant or MPE\textsuperscript{194}. Paramalignant pleural effusions result from tumor effects that indirectly act on the pleural space such as by bronchial obstruction, mediastinal lymph node infiltration, or superior vena cava syndrome; pleural fluid cytology and pleural biopsy are negative. MPE, on the other hand, have positive fluid cytology and/or pleural biopsy for cancer.

When clinicians encounter a patient with a paramalignant or a MPE, they frequently ask:

- What is the prognosis?
- Should the effusion be treated?
- What are the treatment options?
- How do I know which option is best?

In the discussion that follows, we introduce the different approaches of management of MPE\textsuperscript{195}.
Figure 10: Algorithm for diagnostic approaches for patients suspected of pleural effusions

Analysis of pleural fluid includes protein and lactate dehydrogenase of pleural fluids and serum, gross appearance, red blood cell, white blood cell with differential count, pH levels, glucose, amylase, cholesterol, triglyceride, cytology, acid-fast bacilli stain, TB culture, TB-polymerase chain reaction, Gram stain, routine culture, carcinoembryonic antigen and adenosine deaminase of pleural fluids. CHF: congestive heart failure; LC: liver cirrhosis; NT-proBNP: N-terminal pro-b-type natriuretic peptide; TB: tuberculosis; CT: computed tomography.

(From Moon Jun Na, Diagnostic Tools of Pleural Effusion, Tuberc Respir Dis. 2014 May;76(5):199-210)
1.4.2 Prognosis

The median survival among 417 patients identified in a systematic review was four months after the recognition of a MPE, although some patients may have a prolonged survival\textsuperscript{196}. A series of 278 patients referred to a thoracic surgery clinic for management of a MPE found a median postoperative survival of 211 days\textsuperscript{197}. Three factors, a leukocyte count greater than 12 x 10^9/L, a low serum albumin ≤35 g/L, and a low partial arterial pressure of oxygen (PaO\textsubscript{2} ≤9.5 kPa (71 mmHg), independently predicted a worse survival. Median survival was 42 days when all three factors were present, compared with 702 days when none of the factors were present.

Other clinical variables have also been used to predict survival for individual patients (eg, type of cancer, cell type, tumor stage, characteristics of pleural fluid, pleural fluid biomarkers, extensiveness of pleural involvement with tumor, presence of adhesions, and performance level)\textsuperscript{112, 198–199, 200–201}. In contrast, one determined that the prognosis of patients with MPE undergoing talc pleurodesis was independent of age, gender, type of malignancy, and pleural fluid volume\textsuperscript{202}.

The prognosis also depends upon whether the underlying tumor will respond to systemic therapy. As an example, patients with a lymphoma or breast cancer that is responsive to chemotherapy are more likely to have prolonged survival compared to those with an effusion due to non-small cell lung cancer. To date, however, no studies exist to allow sufficiently accurate predictions of survival to assist decision making for management of individual patients with MPE.

1.4.3 Indications for treatment

The decision to use a pleural intervention to treat a malignant effusion depends upon the presence of respiratory symptoms of which dyspnea is the most common and prominent. Some tumor types, such as breast or ovarian cancer or lymphomas, may respond to therapy directed at the underlying tumor with resolution of the effusion.

- Asymptomatic MPE do not need to be treated as long as they remain asymptomatic, although nearly all malignant effusions eventually become symptomatic.
- Symptomatic malignant effusions that do not respond to treatment of the underlying tumor require consideration of palliative therapy directed at the pleural space.
Because successful management of a MPE is palliative and without survival benefit, clinicians should focus on patient-centered goals of therapy, which include sustained symptom relief, improvement in quality of life, patient and family acceptability of an intervention, affordability, and avoidance of invasive procedures and attendant complications that take patients from home and disrupt the course of their remaining life\textsuperscript{203}.

1.4.4 Treatment options

Patients who have symptoms caused by a MPE should initially undergo therapeutic thoracentesis to drain the fluid. An initial thoracentesis does not decrease the effectiveness of subsequent procedures to produce pleurodesis. The rate of reaccumulation of the pleural effusion, the patient's prognosis, and the severity of the patient's symptoms should guide the subsequent choice of therapy\textsuperscript{194}.

Slow reaccumulation

Patients whose MPE reaccumulates slowly (eg, more than one month) may be managed with repeat therapeutic thoracentesis. This is particularly appropriate if patients also have a short (<3 months) expected survival and poor performance status\textsuperscript{204}.

Thoracentesis

Multiple repeat therapeutic thoracentesis is a simple approach to managing MPE that reaccumulate slowly. Briefly, topical analgesia is administered and then a catheter is percutaneously advanced into the pleural space under sterile conditions. A large volume of pleural fluid is then drained. The procedure can be performed at the bedside or in an office setting with appropriate monitoring. Additional details about the technique of thoracentesis are provided separately.

Reexpansion pulmonary edema (REPE) is rare following large volume thoracentesis\textsuperscript{205, 206}. A safe threshold rate or volume of pleural fluid removal that will not cause reexpansion pulmonary edema has not been identified. In our experience, it is generally safe to continue to remove pleural fluid, as long as the procedure is terminated if the patient develops anterior chest pain or the pleural pressure drops below -20 cm H\textsubscript{2}O\textsuperscript{207}. If pleural pressure monitoring is not used, removal of pleural fluid beyond 1.5 L should be accompanied by careful attention to the development of chest symptoms and consideration of the relative merit of removing more fluid for greater symptom relief versus the small potential risk of REPE. Cough is often noted during lung reexpansion and does not correlate with development of REPE.
Repeated thoracentesis may induce pleural adhesions that can complicate thoracoscopic pleurodesis if that procedure becomes indicated. The role for repeated thoracentesis has decreased with the advent of tunneled pleural catheters, which avoid the need for frequent clinic visits for pleural drainage. Other complications of therapeutic thoracentesis are the same as those for diagnostic thoracentesis.

**Rapid reaccumulation**

A more aggressive intervention is required for most patients because the MPE recurs rapidly (eg, less than one month) after an initial thoracentesis. Options include indwelling pleural catheter drainage, pleurodesis, pleurectomy, and pleuroperitoneal shunt.

**Indwelling pleural catheter**

Placement of an indwelling pleural catheter (also known as a tunneled pleural catheter) with intermittent outpatient drainage by the patient or a patient attendant is our preferred initial step for most patients with recurrent malignant effusions. This method is preferred as primary therapy because it is the least invasive option and requires little if any time in the hospital, as the catheter is usually placed during an outpatient procedure. In addition, indwelling pleural catheter drainage is indicated when there is irreremediable lung entrapment or endobronchial obstruction by tumor; in these patients, chemical pleurodesis is contraindicated due to high failure rates when the lung is unable to expand against the chest wall. Indwelling pleural catheters may improve symptoms following a failed pleurodesis. Complications include bleeding and, in particular, infection.

The patient or a family member will need to be able to perform pleural fluid drainage via the indwelling pleural catheter at home and maintain catheter sterility.

Indwelling pleural catheters provide a high degree of symptom relief, as demonstrated in a retrospective analysis of 250 pleural catheter procedures (223 patients). Dyspnea resolved following 39 percent of the procedures and improved in another 50 percent. Additional observational studies have demonstrated that patients who chose indwelling catheters as opposed to talc pleurodesis have fewer total hospital days, fewer effusion-related hospital days, fewer subsequent procedures to control effusion-related symptoms, and better quality of life within seven days of treatment.

Two randomized trials have compared indwelling catheter drainage and pleurodesis in terms of outcomes such as palliation of dyspnea, lung re-expansion, procedure related complications, and quality of life and found similar results. In the larger of two trials, 106 patients with MPE were randomly assigned to placement of an indwelling catheter or pleurodesis with talc slurry via a 12-French chest tube placed over a guidewire. No significant difference was
found in post-procedure dyspnea, chest pain, or quality of life scores between the groups. Twelve patients in the talc group required additional pleural procedures compared with three in the indwelling catheter group (OR 0.21, 95% CI 0.04-0.86). However, adverse events (eg, pleural or skin infection, catheter blockage) were more common in the indwelling catheter group. One multicenter study demonstrated a low incidence of infection (5 percent) from indwelling pleural catheters with an overall mortality risk from infections of 0.29 percent\textsuperscript{213}. Fifty-four percent of the infections in this study could be treated with antibiotics without removal of the chest catheter. Pleurodesis occurred in 62 percent of patients who experienced a catheter-related infection\textsuperscript{213}.

Spontaneous pleurodesis may occur in approximately 50 to 70 percent after two to six weeks of indwelling pleural catheter drainage. In one study, catheter drainage achieved pleurodesis in 70 percent at a mean interval of 90 days after catheter placement\textsuperscript{222}. When drainage ceases, the indwelling pleural catheter can be removed. However, catheter removal is associated with fracture of the catheter in 10 percent with occasional retention of a catheter fragment in the pleural space\textsuperscript{215}. Among four patients with retained catheter fragments, no complications were reported during a mean follow up of 459 days.

For those patients who do not develop a spontaneous pleurodesis after days to weeks of drainage, a pleural sclerosant can be instilled through the catheter\textsuperscript{223} 224 225. For those patients in whom continued drainage is preferred but is prohibited due to catheter-related loculations, the instillation of an intrapleural fibrinolytic agent may be of benefit\textsuperscript{226}.

**Chest tube size**

No convincing advantages derive from performing chemical pleurodesis via a standard large-bore (eg, 24 French) as opposed to a small-bore chest tube (eg, 12 French)\textsuperscript{227} 228 229. However, consistently, large-bore chest tubes cause more patient discomfort. In a randomized trial of 320 patients with MPE, smaller chest tubes (12 French) were associated with a similar rate of pleurodesis failure at three months when compared with patients in whom large chest tubes were placed (24 French) (30 versus 24 percent)\textsuperscript{229}. However, pain scores were significantly lower among patients with smaller chest tubes (mean visual analogue score, 22 mm versus 27 mm), and although the complication rate was higher with the insertion of smaller chest tubes it was not significantly different (24 versus 14 percent). Smaller and older studies have shown a similar lack of difference between small and large-bore chest tubes in this population\textsuperscript{227} 228 230. Methodologic flaws including small sample size for group comparisons and uncertainty in some studies whether the small bore chest tubes were inserted under imaging guidance limit the interpretation of these studies.
Analgesia

The parietal pleural membrane contains a high percentage of pain receptors such that the induction of inflammation by the intrapleural instillation of a sclerosant is often intensely painful. NSAIDS and opiates are frequently prescribed by clinicians for pain control. It is thought that NSAIDS may interfere with the induction of inflammation required for successful pleurodesis, and they are therefore generally avoided. However, the choice of agent is typically individualized and dependent upon factors including level of pain, known sensitivity to opiates or NSAIDS, history of gastrointestinal bleeding, opiate drug abuse, liver or renal failure, and expected poor survival.

In a randomized study of patients with MPE, a similar rate of pleurodesis failure was noted in patients treated with opiates (10 to 20 mg oral morphine four times daily) when compared with patients treated with NSAIDS (ibuprofen 800 mg three times daily) (20 versus 23 percent)\textsuperscript{229}. However, although pain scores were no different between each group, twice as many patients who used NSAIDS required rescue analgesia with intravenous morphine.

Pleurectomy

Radical total or subtotal pleurectomy (resection of visceral and parietal pleura) and decortication (removal of fibrous pleural rind) can control MPE in patients who have failed chemical pleurodesis.

Pleurectomy/decortication can also be used as a primary therapeutic modality for patients with malignant effusions due to mesothelioma, although it does not improve survival and is associated with significant complications\textsuperscript{231, 232, 233}. The management of MPE is discussed separately.

Appropriate candidates must be good surgical candidates and have a reasonably long expected survival, because total radical pleurectomy/decortication requires a thoracotomy and is a major surgical procedure associated with considerable morbidity and some mortality\textsuperscript{234, 235}. Subtotal pleurectomy/decortication can be accomplished thoracoscopically. Pleurectomy is virtually always effective in obliterating the pleural space for control of a MPE.

One observational study reported pleurectomy in 19 patients with MPE performed by single incision thoracoscopic surgery\textsuperscript{236}. Pleural effusions did not recur in 91 percent of patients and no mortality or complications were observed.

Shunt

Pleuroperitoneal shunting is a rarely used option for patients who have lung entrapment, malignant chylothorax, or have failed pleurodesis\textsuperscript{237, 238, 239}. In general, we prefer using an indwelling pleural catheter, rather than a pleuroperitoneal shunt, due to the frequency of shunt-
related problems and the less invasive nature of indwelling pleural catheters. Pleuroperitoneal shunting, however, may have nutritional advantages for patients with malignant chylothorax and high volumes of pleural fluid drainage because shunting of nutrient-rich chyle to the peritoneal space allows its reabsorption.

Usually, the procedure is performed during thoracoscopy and under general anesthesia but may be placed by interventional radiological techniques. The shunt catheter (Denver pleuroperitoneal shunt), has one or two one-way valves that allow unidirectional flow from the pleural space and is inserted with one end in the pleural cavity and the other through a subcutaneous tunnel into the peritoneum; the shunt pumping chamber is placed in a subcutaneous pocket overlying the costal margin.

In the hands of experienced operators, placement of a pleuroperitoneal shunt is reasonably safe, although shunt-related complications occur in about 15 percent. The major problems have been shunt failure, most commonly due to occlusion of the catheter and infection. In a retrospective review of 160 patients who received a pleuroperitoneal shunt for MPE, 12 developed shunt occlusion (requiring revision in 5 and replacement in 7); another 7 developed infection. One patient developed malignant seeding on the chest wall at the site of shunt insertion, but peritoneal seeding was not observed. It is not known whether patients who have experienced shunt occlusion are at greater risk for occlusion after a new shunt is placed.

Palliation of the pleural effusion is achieved in 73 to 90 percent of properly selected patients.

Investigational

Some data suggest that some MPE due to non-squamous subgroup of non-small cell lung cancer (NSCLC) may respond to bevacizumab, an antibody directed against vascular endothelial growth factor.

Intrapleural fibrinolytic agents

Small case series report symptomatic benefit for patients with loculated MPE after intrapleural instillation of urokinase or streptokinase to break down fibrin adhesions and promote drainage. The small size of these studies does not support general recommendations for intrapleural fibrinolytic therapy, which requires careful patient selection and monitoring.

Choosing among the options

Selection of an approach depends on local expertise and practice patterns, individual patient clinical features, and patient preferences. Some of the factors that guide the choice of an approach include the following:

- Indwelling pleural catheters require little if any time in the hospital; however, there is more inconvenience related to the longer dwell time of the tube and slightly greater risk of cellulitis
at the insertion site. In addition, the patient or a family member needs to be able to perform intermittent drainage at home. This option is ideal for patients with a shorter anticipated duration of survival (less than six months), for patients who prefer outpatient management and a less invasive intervention, and for patients who have failed other modalities for pleurodesis. - Talc slurry instilled via small bore chest tube is a reasonable and cost-effective alternative for patients with an expected survival of more than six months\textsuperscript{246 247 248}. The main disadvantage is initial pain with instillation and small but potentially serious risk of respiratory complications. - Intrapleural doxycycline is somewhat less effective than talc and may be associated with more pain. As a result, it would be used in settings where talc is not available. - Video-assisted thoracoscopic talc insufflation is a good choice for patients with longer expected survival, particularly when a pleural malignancy has just been identified during a diagnostic thoracoscopy or when lysis of adhesions or partial decortication is needed to treat lung entrapment. - Combining thoracoscopic talc insufflation with insertion of an indwelling pleural catheter that remains in place until pleural fluid no longer re-accumulates is undergoing investigation, but has not yet been compared with more standard procedures in clinical trials. - Pleurodesis is not indicated in the presence of irremediably entrapped or trapped lung when a therapeutic thoracentesis does not improve dyspnea. Some patients with entrapped or trapped lungs where partial pleural apposition can be achieved after removal of pleural fluid may experience symptomatic relief with pleurodesis\textsuperscript{249}. Other patients with a combination of a completely unexpandable lung and recurrent MPE are best managed with an indwelling pleural catheter. - Pleuroperitoneal shunting is a rarely used option for patients who have failed pleurodesis or have the combination of recurrent malignant effusion, lung entrapment, and also the inability to perform intermittent drainage via an indwelling pleural catheter at home. **Antitumor therapy** Antitumor therapy (ie, chemotherapy, radiation) may be useful as an adjunct in selected situations, but generally is not adequate to control symptoms caused by a MPE\textsuperscript{250 251}. **Chemotherapy** The response of MPE to systemic chemotherapy is disappointing for most malignancies. Exceptions include pleural effusions due to lymphoma, breast cancer, small cell carcinoma of the lung, germ cell tumors, prostate cancer, and ovarian cancer. Preliminary phase I and II studies with nonsclerosant intrapleural chemotherapeutic agents have shown a high rate of control of the malignant effusions, but have not directly compared
these agents to standard therapy\textsuperscript{252,253}. Further randomized trials are needed with comparisons to management with an indwelling pleural catheter alone, thoracoscopy, and/or pleurodesis using traditional sclerosing agents.

\textit{Radiation therapy}

Radiotherapy directed at the primary tumor may be helpful in resolving MPE when mediastinal lymph node disease predominates (eg, lymphoma). Mediastinal radiation also may be effective for resolving paramalignant effusion in patients with lymphomatous chylothorax.

\textbf{Pleurodesis}

The pleurodesis is an other approach that consists in obliteration of the pleural cavity. Therefore it could be considered as 'mechanistic', because it acts without any real action on the mechanism of pleurisy.

Pleurodesis acts against the pleural hyperpermeability caused by tumor. Indeed, the impairment of effective pleural fluid absorption is attributed to tumor-associated blockade of lymphatic outflow. Tumor dissemination in parietal pleural stomata and mediastinal lymphnodes obstructs drainage of fluid from the pleural cavity. Hyperpermeability of pleural vessels seems to be cause also by several vasoactive mediators secreted by tumor cells (vascular endothelial growth factor (VEGF) and angiopoietins, endostatin). Moreover, also host cells can producing these vasoactive and proinflammatory mediators, acting on pleural hyperpermeability, angiogenesis and inflammation (transforming growth factor-\(\beta\) (TGF-\(\beta\), interleukin (IL)-6, tumor necrosis factor (TNF), chemokine ligand 2 (CCL2) and secreted phosphoprotein-1 (SPP100)). In addition, also granulocytes, monocytes, macrophages, mast cells, lymphoid cells, endothelial and fibroblast progenitors interact with tumor cells and seem to have a role in angiogenesis and inflammation, important for MPE pathogenesis. Therefore, MPE pathobiology should include tumor-host crosstalk in the pleural space which dictates the occurrence of vasoactive signaling, host cell recruitment and activation, vascular leakage, angiogenesis, inflammation and tumor dissemination and ultimately MPE formation\textsuperscript{254,255}.

The decision to undergo pleurodesis is often based upon an anticipated survival of longer than three months and the patient's desire for an effective therapy that can be carried out in a single definitive procedure, rather than the potential inconvenience of having a long-term indwelling catheter that requires intermittent drainage if spontaneous pleurodesis does not occur\textsuperscript{256,257,258}.

The type of pleurodesis (chemical versus mechanical), sclerosing agent (talc versus other), chest tube size (large- versus small-bore), choice of analgesic agent (opiate versus nonsteroidal anti-inflammatory [NSAID]) varies considerably among centers and experts. Based upon data derived from retrospective studies and small randomized trials of patients with malignant
pleural effusion (MPE), we prefer chemical pleurodesis using talc slurry via a small-bore chest tube and the avoidance of nonsteroidal anti-inflammatory drugs (NSAIDs) following the procedure. However, for those in whom this option is not feasible, thoracoscopic administration of talc or performance of thoracoscopic mechanical pleural abrasion followed by continued MPE drainage with a large bore chest tube and NSAID administration appear to be viable and effective options.

Chemical pleurodesis refers to obliteration of the pleural space by the induction of pleural inflammation and fibrosis using a sclerosant (usually talc). The sclerosant can be instilled via a chest tube or indwelling catheter (talc slurry) or it can be administered at the time of thoracoscopy or thoracotomy (talc insufflation/poudrage). We prefer chemical pleurodesis via a chest tube rather than via thoracoscopy because the former is as effective but is less invasive and better tolerated. However, thoracoscopic chemical pleurodesis is an appropriate alternative in some patients. Choosing among these options usually depends upon the medical circumstances, goals and preferences of the patient, and institutional practice. As an example, thoracoscopy may be preferred in those with a concomitant indication for thoracoscopy or those in whom diagnosis of pleural malignancy is made during thoracoscopy. In contrast, administration via a chest tube at the bedside may be preferred in patients with poor Karnofsky index who cannot tolerate surgery.

The introduction of sclerosant via small-bore chest tube or during video-assisted thoracoscopy have similar efficacy in studies of patients with MPE due to a variety of cancers, including malignant mesothelioma.

Also sclerosant agents could be used for this aim. Talc is typically the agent preferred by many experts given its relative superiority for reducing the rate of MPE recurrence when compared with other agents in patients with a variety of cancer cell types. In general, the success rate of talc in preventing recurrence is approximately 60 to 90 percent. The longer the patient survives after pleurodesis, however, the greater the probability of recurrence with 50 percent of patients undergoing talc pleurodesis experiencing inadequate fluid control at six months. The tetracycline derivative doxycycline is an alternative sclerosant with reported success rates of about 80 percent.

Studies differ regarding the influence of tumor type on the success rate of pleurodesis. Some studies report no differences, but one retrospective study of 447 patients undergoing talc pleurodesis by thoracoscopy demonstrated a lower success rate for mesothelioma (76 percent) as compared with breast cancer (93 percent). The authors also reported that
pleurodesis success was negatively influenced by high intrapleural tumor burdens, which is especially common in patients with mesothelioma. Another approach is to combine the efficacy and rapidity of pleurodesis with the shortened hospitalization time of an indwelling catheter. The procedure involves complete drainage of the pleural fluid under thoracoscopic guidance, placement of a tunneled pleural catheter, and insufflation of sterile talc (5 g). At the end of the procedure, a 24 gauge chest tube is placed through the port created for the thoracoscope, attached to suction overnight and removed 24 hours later. The indwelling catheter is drained three times a day on the first postoperative day, twice on the second and third postoperative days, and then once daily until the output is less than 150 mL per day. A chest radiograph is then obtained and the tunneled catheter removed if no reaccumulation of pleural fluid is noted. The combination of thoracoscopic talc pleurodesis and indwelling catheter placement was assessed in a series of 30 patients. All of the patients reported improved dyspnea. Successful pleurodesis was noted in 24 of the 26 patients who were still alive at six months; the remaining two still required intermittent drainage using the catheter. The indwelling catheter was removed at a mean of 16.6 days and a median of 7.5 days following the procedure. Adverse effects included fever in two patients, need for replacement of the indwelling catheter due to pleural loculations in one patient, and an empyema in one patient.
1.5 CONCLUSIONS

The most common therapeutic approach for patients with MPE is pleurodesis. Anyway, this treatment provides the obliteration of pleural cavity and consists of a mechanicistic approach. Indeed, it based on the modulation of the genesis of recurrent effusion, very common in malignant pleuresis.

Other innovative approaches are needed also to monitor these pleural effusions and to design new therapeutical strategies.

Moreover, the differential diagnosis of pleural effusion remains difficult and various noninvasive biomarkers proposed nowadays are not sufficiently sensitive to distinguish between benign and malignant pleural pleuresis.

Therefore, the microparticles represent an interesting tool as possible non-invasive markers, to achieve early diagnosis, to monitor the disease, to identify patients benefiting from a specific treatment, and to design new innovative drugs.
CHAPTER 2: GENERALITIES ON MICROPARTICLES

2.1 ORIGIN AND CLASSIFICATION OF EXTRACELLULAR VESICLES

The knowledge of the microparticles (MPs) is not derived from a single discovery that has demonstrated their presence regardless of their cell of origin. In fact, the findings in this domain concerns several independent and focused studies on a certain cell subset.

The microparticles have been recognized in different ways and the scientific discovery was highly variable: sometimes from recognition of a loss of membrane material, sometimes as precipitate, or through mere microscopic observation.

The link between these entities has finally been made relatively late in the 1990s. This awareness of the universality of cell vesiculation phenomenon has led to the extension of this research to a multitude of biological media and cell types.

2.1.1 Hematopoietic and vascular cells

The notion that the aging and destruction of red blood cells are associated with a loss of phospholipids and cholesterol is very old. The first mention of MPs was made in 1949. Erwin Chargaff and Randolph West reported for the first time the existence of a precipitated factor in a plasma wafer which can accelerate the generation of thrombin. At that time this discovery was contrary to established dogma by which platelets were essential to the cascade of blood coagulation.

Chargaff and West also found that the ability to clot with a high-speed plasma centrifuged (31000g) was significantly decreased. They had concluded that the presence of a procoagulant plasma component, found in the pellet of a high-speed centrifugation.$^{273}$
In 1955, Hougie found that the procoagulant activity of a citrate platelet rich plasma increased significantly during sample storage. Hougie had attributed these potential results at procoagulant platelets\textsuperscript{274}.

In the same year, O'Brien reported that the clotting time of a platelet rich or poor plasma was shortened when it added the serum.

Following these comments, a question remained insoluble, whence came the common procoagulant activity in platelet-poor or rich plasma and serum?

More than a century later, Weedet et al. proposed the fragmentation of red cell membrane as in vivo and in vitro mechanism of this loss of phospholipids\textsuperscript{275 276 277 278}.

In 1964, Michelet et al. reported the presence of specific material in stored blood plasma\textsuperscript{279}.

In 1969, Cooper and Jandl described the loss of cholesterol and phospholipids by red blood cells incubated in deprive serum glucose, but he did not suspect the phenomenon of vesiculation\textsuperscript{280}.

The first to use this term to describe the production of vesicles incorporating red cell membrane sialoglycoproteins was the MacDonalds in collaboration with Martin\textsuperscript{281 282 283}. At the same time, Bessis and Mandon published microscopic pictures of microspheres (Fig. 11A)\textsuperscript{284 285}.

In 1967, a British physicist, Peter Wolf, helped bring the missing piece to previous observations. His article "The Nature and Significance of Platelet Products in Human Plasma", published in the British Journal of Haematology, described the existence of material derived from cells found in the membrane of ultracentrifugated pellet and able to generate thrombin\textsuperscript{286}.

These "platelet remains" obtained from citrated plasma or serum have a procoagulant potential identical to platelet factor 3, now called tissue factor (TF), responsible for the conversion of prothrombin into active form: thrombin\textsuperscript{287}. Wolf was also the first to describe the existence of a linear correlation between the rate of platelet-derived MPs (MPPs) (he called at the time "platelet dust") and the platelet count in a blood sample. He reported a high level of platelet-MPs in patients with thrombocytosis and low in thrombocytopenic patients. Following these discoveries, Peter Wolf developed an interest for these "platelet residues" that will be called microparticles.

The new technologies such as electron microscopy, offered the possibility for other teams to confirm the existence of small sub-micron vesicles released by circulating platelets. Many expansions were observed for activated platelets with budding at the distal end (Fig. 11B)\textsuperscript{288}.

In 1985, vesicles from granulocytes, named ectosomes were identified. They were then associated with a cellular response to mechanism of complement (Fig. 11C and 11D)\textsuperscript{289 290 291}. 

58
In 1994, Satta et al. disclosed the existence of circulating MPs derived from monocytes and THP-121 cells. They reported that MPs could be generated after stimulation of monocytes with lipopolysaccharide (LPS). The same team describe three years later the MPs derived from lymphocytes in the context of the HIV virus (human immunodeficiency).

Hamilton and al. described for the first time in 1990 a procoagulant activity associated with the culture supernatant of endothelial cells from human umbilical vein (HUVEC) and reported the presence of membrane elements capable of generating thrombin in the presence of complement.

It was in 1999 that our team first described endothelial microparticles (EMPs) and their morphological, phenotypic and functional features. In the same study, the in vivo proof of concept of the existence of these EMPs has been made in healthy individuals and in patients with associated complications of antiphospholipid syndrome.

We showed by electron microscopy that the process of budding on the surface of HUVECs, stimulated by TNF, leads to the increase in the number of MPs released (Fig. 11E).

### 2.1.2 Tumoral cells

Initial observations of tumour-derived MPs (TMPs) at electron microscopy were unintentionally obtained, and the authors were themselves not particularly aware of the presence of these vesicular bodies.

For example, tumour vesicles are clearly visible on murine teratocarcinoma images 1962 or induced renal tumour in the rat in 1967.

Tarin is the first author, who called the MPs "vesicular structures" or "fragmented material".

In the following decades, these vesicles were isolated from cultured murine tumour cell lines supernatant, ascites fluid, of patients suffering from ovarian cancer and villous adenoma secretions (Fig. 11F).

Very quickly, the interest has focused on the relationship between these fragments and cancer metastasis.

### 2.1.3 Other cell types

The vesiculation phenomenon is a process common to all cells.
Since the first discoveries previously mentioned, various MPs subpopulations derived from other cell types have been described and studied such as MPs from chondrocytes, oligodendrocytes, fibroblasts, syncytiotrophoblasts, podocytes, adipocytes, smooth muscle cells, megakaryocytes, neuroepithelial cells, embryonic stem cells, mesenchymal stem and hepatocytes\textsuperscript{304 305 306 307 308 310 311 312 313 314 315 316 317}.

After these experiments on culture supernatants and plasma, other biological media were used to characterize the MPs as urine, ascites fluid, cerebrospinal fluid, atherosclerotic plaque, synovial fluid, bronchoalveolar fluid, muscle, vitreous humour, saliva, amniotic fluid the pleural fluid, and mucus nasal tears\textsuperscript{318 319 320 321 322 323 324 325 326 327}.

These numerous studies, initially purely descriptive, carry out \textit{in vitro} on cellular purified models, and \textit{in vivo} in different clinical settings, allowed to gradually characterize the structural and functional features of these MPs.

Meanwhile, MPs derived from prokaryotic cells growing interest in literature. Indeed, as well as eukaryotic cells, bacteria (gram + and gram -) are able to produce and secrete MPs (called OMV, outer membrane vesicles), which could have a role in the lipopolysaccharide dissemination of bacteria and in the resistance to antibiotics\textsuperscript{328 329 330}.
**Figure 11: Historical images of microparticles by microscopy**

B. Platelets microparticles (*From George JN et al. Blood 1982*)
E. Endothelial microparticles (*From Combes et al. J Clin Invest 1999*).

### 2.1.4 Classification

In 2005, four decades after the first description by Peter Wolf, a committee for Vascular Biology standardization was created within the International Society of Thrombosis and Haemostasis (ISTH) to set up the nomenclature and standardization to provide recommendations on the use of MPs in clinical practice\(^{331}\). In 2010, the nomenclature was widely adopted during a workshop by members of the International Society of Extracellular Vesicles (ISEV) that has done a considerable work to clarify it\(^{332}\).

This society consists of many scientists from different backgrounds who aim to propose a consensus on different classes of extracellular vesicles. ISEV proposed the term extracellular vesicles to designate all vesicles made by a cell, regardless of their formation mechanism or composition\(^{333}\).

An heterogeneity of these vesicles was defined according to several criteria: size, density, morphology, lipid composition, protein composition and their cellular origin (Table 7).
### Table 7: Characteristic of cellular microparticles

<table>
<thead>
<tr>
<th></th>
<th>Exosomes</th>
<th>Microparticles</th>
<th>Apoptotic bodies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>30-100 nm</td>
<td>0.1-1 μm</td>
<td>0.5-4 μm</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td>Round</td>
<td>Heterogeneous</td>
<td>Heterogeneous</td>
</tr>
<tr>
<td><strong>Markers</strong></td>
<td>CMH, CD81, CD82, CD53, Rab, GTPase, Alix, Tsg101, HSP</td>
<td>MPP: CD42b, CD31, CD41/CD61, CD62P</td>
<td>Histones DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MPEry: CD235a</td>
<td>MPP: CD45, CD14, CD11a, CD11b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MPL: CD31, CD51, CD54, CD62E, CD195, CD146</td>
<td></td>
</tr>
<tr>
<td><strong>Expression of PS</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td>Erythrocytosis of multivesicular bodies</td>
<td>Vesiculation of the plasmatic membrane</td>
<td>Cellular death</td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td>Proteins, mRNA, miRNA</td>
<td>Proteins, mRNA, miRNA</td>
<td>Proteins, mRNA, miRNA, DNA</td>
</tr>
<tr>
<td><strong>Method of detection</strong></td>
<td>Centrifugation &gt; 100 000g Density: 1.13-1.19 g/ml</td>
<td>Centrifugation &lt; 100 000g</td>
<td>Centrifugation &lt; 100 000g</td>
</tr>
</tbody>
</table>

MPs are part of a general classification of extra-cellular vesicles termed microvesicles, which includes a population of membrane vesicles that are heterogeneous in shape and isolated from biological fluids or conditioned culture media\textsuperscript{334}. Other extracellular vesicles include apoptotic bodies and exosomes, which differ on the basis of their size and origin. The irregularly shaped
apoptotic bodies are released from cells undergoing apoptosis and fragmentation, whereas, exosomes are released by the fusion of multivesicular bodies with the cell membrane. The nomenclature is still controversial: presently they are collectively called membrane vesicles.

The term “exosome” comprises vesicles smaller than 100 nm, while the larger ones are commonly referred to as “microvesicles”, “ectosomes”, “microparticles” or “exovesicles”.

Given their overlapping physicochemical properties, exosomes and other types of shedding vesicles are not well distinguished by most purification methods. Thus, there is confusion in naming secreted vesicles, some authors use the term ‘exosome’ according to the stringent criteria, whereas others use the terms ‘exosome’ and ‘microvesicle’ interchangeably. Further terminology problems arise from the confusion with apoptotic blebs, which are larger types of vesicles (50–5000 nm diameter) released by dying cells.

The imperfect consensus is to use the term "exosome" for small vesicles, "apoptotic bodies" for large vesicles and admits "microvesicle" for midsize vesicles (Figure 12).

From a historical perspective, the first scientists used the term "microparticle" when describing the role of platelet MPs in hemostasis while in other areas such as immunology, scientists instead used the term "microvesicles" to avoid confusion with other types of particles (synthetic, silicone ...).

I have chosen to maintain this term MPs throughout the manuscript. Therefore, theoretically, the MPs are defined by their subcellular origin, formation mechanism, size, protein composition and their mode of production (Table 7).

**Microparticles**

MPs are small vesicles 0.1-1μm resulting from remodelling of membrane phospholipids of all activated or apoptotic cells. They consist in/of a phospholipid membrane and they express on their surface phosphatidylinerine (PS) and characteristic antigens of original cell, allowing their identification and contributing to their multiple biological functions.

They do not contain core but nucleic acids and cytoskeletal residue. They are found in the pellet of a rapid centrifugation of platelet-depleted plasma or culture supernatant. At present fortune, the definition of criteria for their precise size and expression of PS are still debated.

**Exosomes**

On the other hand, the exosomes are defined as small vesicles with a diameter of 30-100nm. The exosomes consist of a lipid bilayer and their density varies between
1.13 g/ml to 1.19 g/ml. From intraluminal vesicles formed during the maturation of these endosomes, some of these multivesicular bodies can fuse with the plasma membrane permitting the secretion of exosomes.

They were reported for the first time in 1983 by Johnstane et al. from culture of reticulocytes. They differ from MPs not only by their size and their origin but also by their composition. They did not contain PS and they are enriched in molecules of the family of tetraspannins (CD63, CD81, CD82, CD53…), in molecules of the major histocompatibility complex, and other specific proteins associated with the endosome (Rab GTPase, annexins), some of which are involved in the genesis of multivesicular bodies (Alix, Tsg101).

In contrast to the MPs, an ultracentrifugation of 100000 g or more is required for their isolation.

Their regular and rounded shape allows to isolate them in a sucrose gradient in a low-density area 1.10-1.21 g/mL, which differentiates them from other more irregularly shaped vesicles.

**Apoptotic bodies**

Apoptotic bodies are larger than the MPs (0.5-4 microns). They represent the terminal stage of apoptotic cells. They are generated by fragmentation of the apoptotic cell, which explains the presence of leavings of nuclear material such as DNA and histones. It is not always easy to distinguish them from MPs, because the apoptosis phenomenon can be also source of MPs.

However, it is necessary to emphasize, the actual lack of specific markers for characterizing each class of MPs. In fact, most studies argue the specificity of each criterion and they called into doubt, which leads us to wonder in 2016 if there is a specific marker for each vesicle.
Figure 12: Schematic representation of the release of different microvesicles from cell in the cell-free extracellular space.

A. Exosomes and microparticles.


(From Graça et al. J. Cell Biol. 2013)
2.2 COMPOSITION

MPs are mainly composed of lipids, proteins and nuclear material. Their composition varies according to the cell of origin and the stimulus which triggered their formation.

2.2.1 Lipids

As we already mentioned, MPs are composed of a phospholipid bilayer from the original cell. These phospholipids (PLs) are asymmetrically distributed at rest; this asymmetry is alternated during formation of MPs, which lead to exposure of negative charged PLs such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the external layer\(^{357}\).

MPs composed of about 60% of phosphatidylcholine, the remainder consisting of sphingomyelin, PE and PS\(^{358}\).

Fourcade et al. showed that the phospholipid composition of MPs from synovial fluid of patients suffering from rheumatoid arthritis is different from which of MPs from plasma of healthy subjects\(^{359}\). In the synovial fluid MPs are mostly derived from leucocytes\(^{319}\). This observation confirms that the phospholipid composition of MPs varies with the cell of origin but also by the stimulus that caused their formation. Huver et al. reported that the degree of oxidation of MPs phospholipids also dependent on the stimulus used to induce their vesiculation\(^{360}\).

2.2.2 Proteins

MPs expose different specific membrane antigens of the original cell: cytoadhesines (including integrins), molecules of the major histocompatibility complex, enzymes, glycoproteins, tissue factor (TF) etc.

The presence of glycoproteins of membrane representative of parent cell allows identifying the origin of MPs (platelets, endothelial cells, leukocyte and erythrocyte)\(^{361, 362}\). For example the MPPs express GPIb (CD42b), the platelet endothelial adhesion molecule (PECAM-1; CD31), integrin αIIbβ3 (GPIIb-IIIa), P-selectin (CD62P), CD63, CD41a and CD61.

EMPs express CD31, , 51, 54, 62E (E-selectin), CD62P (P-selectin), CD105 (Endoglin) and
CD146 (S-ENDO1). An example of EMP composition is schematically in Figure 13.

Figure 13: Schematic representation of the panel of molecules conveyed by EMP and the associated biological effects.

EPCR indicates endothelial protein C receptor; PECAM-1, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1; E-selectin, endothelial selectin; S-Endo, CD146/melanoma cell adhesion molecule; VE-cadherin, vascular endothelial cadherin; eNOS, endothelial NO synthase; MMP, matrix metalloproteases; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; EPC, endothelial protein C; TM, thrombomodulin.

(Adapted from Dignat-George et al. Arterioscler Thromb Vasc Biol. 2011).

Knowledge about molecules vectorised by MPs was enriched by data provided by several proteomic studies.
Thus, hundreds of proteins vectorised by MPs have been identified from different subpopulations: plasmatic MPs, EMPs, PMPs, lymphocytic MPs, adipocyte MPs and MPs from red blood cells. These works helped to highlight different types of molecules on the surface of MPs as inflammatory proteins, adhesion molecules, antigen-presenting molecules, cytoskeletal proteins, enzymes or molecules signalling. This information on the protein composition of MPs allow a better understanding of functional properties of these vesicles. Several studies have shown that MPs derived from tumour cells (lung, colon carcinoma, prostate) exhibit a panel of molecules involved in tumorigenesis, including the migration, invasion, modulation of the immune system, angiogenesis, and metastatic process. These data may also facilitate the identification of markers of a pathological context.

The MP is not a copy of the cell from which it is derived. Indeed, the antigenic profile of the parent cell may be different independently from their activation, in particular by the transfer of molecules.

In addition, circulating soluble antigens from other cell types can adhere or fuse with the membrane of MPs. MPs can thus express "adopted" antigens different from their original cell.

Other proteins present in lipid rafts of red cell membrane seem concentrated to the MPs during the vesiculation process compared to other proteins. In fact, the antigen density of FT and PSGL-1, that are in the lipid rafts, is increase at the surface of the MPs compared to the monocytes from which they derive.

In contrast, CD45 which is absent from these microdomains is better expressed on the surface of monocytes. In general, it has been found that the Glycerophosphoinositol (GPI) anchored molecules were easily found on the MPs. Indeed, the weak attachment to the cytoskeleton contribute to enrich these molecules on MPs when the vesiculation process occurs.

Furthermore, the nature of the original stimulus of formation of MPs modified the antigenic profile of MPs. For example, after an inflammatory stimulus, the EMPs are rich in E-selectin, an inducible cytoadhesine involved in the "rolling" of leukocytes on endothelium. When EMPs are expelled from the endothelium by apoptosis they are particularly rich in CD31, a protein constituent of the endothelial cell.
2.2.3 Nucleic acids

During budding process, MPs recover a portion of cytoplasm of the emitting cell, which may contain cytokines, chemokines, enzymes, growth factors and proteins of signalling. This list was supplemented with the identification of functional mRNAs and microRNAs (miR). The miRs are a class of small RNAs (18-25 nucleotides), non-coding, highly conserved, able to down-regulate gene expression by inhibiting translation target RNA or accelerating their degradation.

Thus, the miRs participate in the regulation of biologic processes such as cell differentiation, proliferation, apoptosis, hematopoiesis, carcinogenesis and angiogenesis.

The miRNAs target more than 5300 human genes, representing over 30% of all our genes. In this circulation, miRs are transported and protect against RNAs by transporters like MPs, exosomes, apoptotic bodies, lipoproteins (HDL or LDL) and a small part may be linked to proteins (Figure 14).

For example, in the MPs, the miRNA-223 has been described as controllers of maturation, proliferation and differentiation of myeloid and lymphoid cells.

Moreover, as demonstrated by Diehl et al, the profile of miRNAs from MPs differs from their original cells, suggesting the existence of selective carriers of miRNA during the vesiculation process. The main category of vesicle containing miRNA is subject to debate.

Figure 14: Different miRNAs secretion patterns
It is important to note that these differences of composition, strictly dependent from the initial stimulation and the origin cell, determine also the functional profile of different MPs subpopulations.
2.3 MECHANISMS OF FORMATION

The knowledge about the mechanisms of vesiculation, is mainly based on in vitro experiments, showing the ability of cells to emit MPs in response to various stimuli, such as thrombin, inflammatory cytokines, lipopolysaccharide, reactive species of oxygen (ROS), inhibitor of plasminogen activator (PAI-1), low density lipoprotein (LDL) or uremic toxins. The formation mechanism involves two types of events: a reshuffle of phospholipids (PLs) of the plasma membrane and cytoskeletal rearrangements that lead to cell budding.

2.3.1 Remodelling of the plasma membrane phospholipids

The plasma membrane of eukaryotic cells is composed of a bilayer of phospholipids in lamellar structure also including cholesterol and protein (transmembrane or associated by glycosyl-inositol phosphate anchors). The phospholipids are predominantly glycerophospholipids but also sphingosine. Within the plasma membrane, the presence of cholesterol provides mechanical stability of the membrane, which is about 5 nm thick. The plasma membrane is comprised of about 50% of protein (100 kDa medium) moving in 50% of lipid (0.7kDa in average).

At rest, the membrane is characterized by an asymmetric distribution of phospholipids (PLs) of the lipid bilayer. Cationic phospholipids (phosphatidylcholine and sphingomyelin) are located predominantly at the outer leaflet of the membrane. Conversely, anionic phospholipids (phosphatidylethanolamine (PE) and phosphatidylserine (PS) are concentrated at the inner leaflet. The membrane fluidity results from lateral movement in the plane of the membrane providing the reorganization of some macromolecular series (receptors, ion channels, ...).

The distribution of lipids membrane is the result of an active process (transverse diffusion or "flip-flop") dependent from phospholipid carriers controlling translocations either inwards (flip) or outward (flop). This asymmetric distribution is under the control of the membrane bound ATP-dependent enzymes; flippase and floppase, as well as the ATP-independent transporter scramblase (Fig. 15).

Flippase (the aminophospholipid translocase) rapidly directs phospholipids, specifically PS and PE, to the inner leaflet, whereas floppase promotes the much slower outward translocation of lipids.
Scramblase is a bidirectional transporter and promotes random distribution of phospholipids across the plasma membrane bilayer. Membrane reorganization can occur under normal physiological conditions or in response to stimuli, such as following exposure to proinflammatory or prothrombotic substances, and involves the calcium-dependent inhibition of flippase and the promotion of scramblase and floppase activity. During this process, phospholipids, such as PS, are redistributed from the inner leaflet of the plasma membrane to the outer leaflet (Fig. 16).

Figure 15: Cytoskeleton disruption.
Activated Calpain cleaves the cytoskeleton, leading to the formation of a membrane bleb and subsequent TMP release.

(From J Pharm Pharmaceut Sci (www.cspsCanada.org) 16(2) 238 - 253 2013)
Figure 16: Floppase, Flippase and Scramblase.
A) Schematic of a cellular membrane at rest. Phospholipid asymmetry is under the control of active flippase, whilst floppase and scramblase remain inactive.
B) Cellular activation. Calcium is released from the endoplasmic reticulum, which can lead to the loss of phospholipid asymmetry and the activation of calpain
(From J Pharm Pharmaceut Sci (www.cspsCanada.org) 16(2) 238 - 253 2013)

These carriers are grouped into three groups according to their lipid specificity, transport direction and dependence: the flippase, the floppase and scramblase (Figure 16)\textsuperscript{411}. The presence of PS or PE on the extern layer causes a reverse transport on the inner layer against their concentration gradient by aminophospholipid translocase ATP-dependent and ubiquitous, flippase, now identified as a P4-ATPase\textsuperscript{412} \textsuperscript{413} \textsuperscript{414}. This enzyme has been described for the first time in 1984 by Devaux et al., who measured ATP-dependent capture of lipid analogues by human erythrocytes\textsuperscript{415}. The stoichiometry of this transport is approximatively the
same of an ATP molecule consumed by transported lipid. This activity is inhibited when the intra-cytoplasmic calcium concentrations are of the order of micromole.

Another ATP-dependent enzyme called floppase directs a rapid phospholipids translocation towards the external leaflet of the plasma membrane in a nonspecific manner. Its function is not well understood. It probably works in conjunction with the aminophospholipid translocase. These two enzymes work permanently in quiescent cells, their combined activities can react to the lipid alterations of the plasma membrane. In red blood cells, the floppase activity is carried by the MDR1 protein (Multidrug Resistance Protein 1) belonging to the superfamily of ABC (ATP-binding cassette). The ABCA1 gene (member of the family of ATP-binding cassette transporters ABC), involved in the reverse cholesterol transport, has also been proposed as a key element of the outsourcing of PS in the activated or apoptotic cell. Indeed, erythrocytes of ABCA1-KO mouse outsource PS with reduced efficiency after cell activation. Albrecht et al. have identified a mutation in the ABCA1 gene in an English patient with Scott syndrome, but it has not been found in other family members.

Finally, scramblase is a phospholipid carrier, nonspecific bidirectional activated calcium flux. There is an autosomal hereditary disease recessive disorder associated with moderate haemorrhagic manifestations and characterized by an underlying deficit of scramblase activity on activated platelets and red blood cells and lymphocytes, called Scott syndrome. This anomaly occurs by sequestration of procoagulant phospholipids in the inner leaflet after platelet stimulation. Consequently, the fixation of coagulation factors and their assembly to the platelet surface are decreased, the thrombin is then not produced in sufficient quantities and normal haemostasis cannot be ensured. The study of this functional deficit exposure of phospholipids and emission of MPs was a good opportunity to identify the molecular nature of scramblase activity.

Recently, it has been shown that it belongs to a family of transcription factors bound to the membrane and it was identified as the anoctamine TMEM-16F (transmembrane protein-16F also called anoctamin 6). It allows a specific transfer of PLs according to their concentration gradient. It is present in the platelet membrane thereby quickly and locally initiate the clotting cascade at the vascular injury site. It is also present in the membrane of red blood cells but weaker and less effective amount. The TMEM-16F is a membrane protein consisting eight transmembrane domains whose gene is located on chromosome 12. Several groups have described mutations in the gene encoding this protein in patients Scott syndrome.
Furthermore, Brooks et al. have located on chromosome 27 the deficit area in canine model Scott's syndrome, which is a region similar to the human chromosome 12 where is located the gene encoding the protein TMEM-16F.\textsuperscript{436} The PS exposure and cell vesiculation are closely related events, this association is illustrated by Scott syndrome. As described above, the underlying abnormality is a fault of scramblase activity that leads to reduce the PS expression and production of MPs.\textsuperscript{428} Platelet aggregation to epinephrine, ADP (adenosine diphosphate), to collagen, to ristocetin and the arachidonic acid remains normal while the generation of thrombin is abnormal.\textsuperscript{422, 423} A similar failure with normal pro-thrombinasis has also reported by Castaman et al.\textsuperscript{427}

![Figure 17: Regulation of lipid asymmetry in biological membranes.](image)

The distribution of lipids is provided by three families of carriers like ABC transporters, the P4-ATPases and scramblases. Most of the ABC transporters catalyse by ATP the transport of lipid to the outer leaflet in the membrane lipid bilayer, while P4-ATPases act in the opposite direction. ABC transporters may allow the efflux of lipids to lipoproteins such as Apo-A1 in the case of ABCA1 or to the bile micelles. Some ABC transporters may behave as importers or flippases carrying phospholipids in the same direction as the P4-ATPases. The scramblases are not dependent on ATP and act by suppressing the asymmetry by random distribution of lipids within the two layers of the membrane (PC: phosphatidylcholine, PS: phosphatidylserine, PE: phosphatidylethanolamine; SM: sphingomyelin; GSL: glycosphingolipid; chol: cholesterol)

(From Coleman et al extract BiochimBiophys Acta.2013).

The cell apoptosis or activation are associated with an increased rate of intracellular calcium. This inhibits the activity of the amino-phospholipide-translocase and active the floppase and the scramblase.\textsuperscript{427}
Other mechanisms may lead to the outsourcing of the PS and the release of MPs as the enrichment of cholesterol on monocyte membrane, which then causes the release of MPs of monocyte charged in tissue factor\textsuperscript{437, 438}. These dynamic membrane structures are domains enriched in cholesterol and sphingolipids, capable of assembling or dissociating, depending on the state of cell activation and the response to different stimuli. These microdomains are used to package certain regulatory proteins and to recruit essential molecules in the redistribution of PS. Kunzelmann et al. demonstrated that the integrity of these microdomains is necessary for intracellular calcium mobilization and phosphatidylinerine externalization\textsuperscript{439}. They allow the operation of ROCE-1 calcium channel. Moreover, the externalization of PS depends on the activation of the ERK kinase pathway associated with these microdomains in the inner leaflet. Finally, these areas of the membrane are enriched in GPI (glycero-Phospho-Inositol) anchored which are enriched on MPs\textsuperscript{440}.

Some studies also emphasize the importance of the reuptake mechanism of extracellular calcium as modulator of cytosolic stock and of the exposure of PS after platelet activation: the SOCE (store-operated calcium entry)\textsuperscript{441}. This mechanism is mediated by membrane channel proteins of the family of Trp\textsuperscript{442}. The importance of this mechanism has recently been shown in an animal model. Mice platelets expressing a mutated form of Orai1 (a part of a channel SOCE) show a PS exposure defect after stimulation\textsuperscript{443}.

On the other hand, the inhibition of phospholipids randomization suppresses vesiculation of red blood cells induced by calcium\textsuperscript{444}. At this level, one can recognize that not all actors involved in the maintenance or in the breaking of the asymmetry of the membrane have been accurately identified\textsuperscript{445}.

This can be explained in part by the technical difficulties accompanying experiments modulation of the composition of phospholipid membranes. At present, no PS internalization defect has been reported, making uncertain the identity of the aminophospholipid translocase. Several candidates for floppase activity have been proposed, but none has been confirmed by lack of specific tests. It also seems that several mechanisms exist based on the induction mode. Moreover, it appears that several mechanisms of outsourcing PLs are possible, depending on the origin and type of stimulus (cell activation, apoptosis) and calcium is necessary every time. There appears to be other alternative ways of TMEM-16F protein, involved in moderate haemorrhagic manifestations patients Scott syndrome\textsuperscript{434}. 
2.3.2 Reorganization of cytoskeleton

The integrity of the cytoskeleton also helps to maintain the asymmetry of membrane and cell shape. Its reorganization is involved in membrane budding of activated cells (Figure 18).

**Figure 18: Key players in the remodelling of the cytoskeleton and vesiculation.**

The phospholipids present an asymmetric distribution (left), in particularly the aminophospholipids (red), PS and PE are localized to the inner leaflet. The reorganization of cytoskeleton involves protease activated by cytosolic calcium such as caspases and calpains. An increase in intracellular calcium is necessary for the PS exposure and involves different mechanisms: transmembrane pores and SOCE. The rearrangement of the cytoskeleton leads to budding of the membrane and release of MPs.


This concept was originally suggested by the discovery of contractile proteins in the PMPss by Crawford et al in 1971. The work of Fox and al. showed that the cytoskeleton stabilizes resting platelets and prevents vesiculation. In other cell types, detachment of the plasma membrane of cortical actin cytoskeleton is associated with the protrusions formation at the
level of membrane and with vesiculation.  

**Calpains**

Calpains are cysteine proteinase calcium dependent of the papainases family. They regulates cytoskeletal proteins, has also been reported as a component of the TMP biogenesis machinery in platelets. Their role has been demonstrated using inhibitors, such as calpeptin, capable of partially prevent formation MPs. Moreover, the presence of active calpain was demonstrated by western blot in MPs from activated platelets. Their activation needs a dozen of calcium ions for each molecule to induce outsourcing PS. In thrombotic thrombocytopenic purpura (TTP), plasma calpain activity is highly correlated with the level of PMPs. The activated calpains induce proteolytic cleavage of cytoskeletal proteins such as filamine-1, gelosine, talin and myosine. They also activate apoptosis through Bcl-procaspase3 and Bcl-xL.

**Gelsolin**

Gelsolin is present in platelets. It is specific for the formation of PMPs. It is induced by the cytosolic calcium and cleaves chaperones like adducin and Z cap at the end of actin filaments of the cytoskeleton during platelet activation, allowing the cytoskeleton remodelling of actin.

**Molecular mechanisms of cytoskeletal remodelling**

The molecular mechanisms of cytoskeletal remodelling resulting in loss of adhesion between the cytoskeleton and the plasma membrane have been defined. The actin-myosin interaction and the maintaining of a flexible cytoskeleton in response to external stimuli are delivered through the ROCK-I protein.

ROCK-I is a Rho-kinase activated by a small protein GTPase, Rho. It belongs to the Ras superfamily. ROCK-I acts by phosphorylating the myosin light chain (MLC), which generates contractile forces by micro-filaments of cytosqueleton.

The apoptosis cell leads the activation of caspase 3. Caspase 3 has an important role in the morphological changes in the cell membrane. ROCK-I is then cleaved by caspase 3 and thereby releases its carboxyl-terminal inhibitory domain. ROCK-I is not regulated but remains active and phosphorylates the more MLC.

Thus, the contraction of actin and myosin filaments causes the detachment from the membrane.
and a budding and release of vesicles.

ROCK-I is not the only signalling molecule to be involved in consecutive vesiculation to phosphatation MLC.

Muralisharan-Chari et al. have shown in a model of tumour cell that the GTP-binding protein activates ERK ARF6, which induces phosphorylation of MLCK and therefore the MLC phosphorylation. This facilitates the actin-myosin contraction at the site vesiculation and leads to the formation MPs\(^{463}\).

Sapet et al. demonstrated new group of genes involved in the MPs generation by endothelial cells stimulated by thrombin: Rho-kinase ROCK-II by activated caspase 2\(^{464}\).

### 2.3.3 Cell activation

Vesiculation is involved in physiological or pathological turnover membranes cell. If it occurs at a basal state, the vesiculation can be amplified during different biological processes such as cell differentiation, stress or activation cell, stimulation by cytokines, shear forces, senescence or apoptosis.

MPs are released constitutively and/or in response to activation signals. They are generated from cells by two distinct mechanisms\(^{408,465}\).

MPs, smaller than 100 nm, are formed by exocytosis, thus the term “exosome”. The first step in the process is the assembly of several endosomes into a multivesicular body. After exiting the lysosomal pathway, this structure fuses with the plasma-membrane. Cytoplasmic proteins can enter the future exosomes as well, which together with the proteins of the invaginated cell membrane represent the “donor cell”. Thus, the origin of the exosomes can be identified through the specific protein set of the donor cell. The sorting mechanism of the selected proteins remains poorly understood\(^{466,467}\). Vesicles, shedding from tumor cells, fall into the exosome category in majority.

MPs between 100 and 1000 nm are generated by a different process, called “reverse budding”. Briefly, either activating signals or an increase of intracellular Ca\(^{2+}\) ion concentration initiate the reorganization of the cytoskeleton and of the membrane, which leads to vesicle formation\(^{408,467}\) (Fig.19).
Figure 19: Schematic drawing of the formation of exosomes and MVs.

Exosomes are released via exocytosis. Cytoplasmic proteins enter the vesicles during their “maturation” in the endocytotic – multivesicular body pathway. Larger MVs shed via reverse budding.

(From E. Pap et al. / Critical Reviews in Oncology/Hematology 79 (2011) 213–223)

Microvesicles can be released from different cells, including red blood cells, platelets, lymphocytes, dendritic cells, fibroblasts, endothelial cells, and epithelial and tumor cells. Recent reports suggest that different types of MPs can originate from the same donor cells, and whether the various biogenetic pathways are completely independent or overlapping, and to what extent, needs further study.

Platelets

Our study focuses on PMPs, knowing that platelet activation is necessary (but not always sufficient) and closely coupled to the formation of these vesicles. The effect is particularly marked when platelets are activated by calcium ionophore (A23187, ionomycin) and the association thrombin/collagen. It is more moderate in response to collagen, thrombin, ADP or epinephrine. Furthermore, some of these agonists may have additive or synergistic effects, e.g. platelet activation is potentiated by C3a and ADP association. The mechanism of vesiculation induced by collagen is mediated by phosphorylation of p38 MAPK. The great variability of the effect of agonists, suggesting that platelets activation is a complex process. The membrane attack complex terminal is a potent inducer of platelet vesiculation in vitro. The
work of Sims et al. and the study of Wiedner et al. showed that the stimulation of platelets by sub-lytic concentrations of C5b9 induces platelet activation with secretion of dense granules and alpha granules, associated with release of membrane vesicles. Indeed, by studying potential changes membrane of platelets, they discovered in 1985 that C5b-9 can cause rapid depolarization followed by repolarisation. MPs seem specifically detached from the membrane sites in which the membrane attack complex (CAM) is insert, dependently of extracellular calcium penetrating through the cellular pores created by the CAM. In vivo, activation of complement is likely involved in platelet vesiculation during autoimmune diseases such as autoimmune thrombocytopenic purpura (ITP). Indeed, Horstman et al. have shown that opsonized platelets by monoclonal antibodies directed against the GPIIbIIIa release MPs in the presence of serum as a complement source. This effect is inhibited by heating the serum for 30 min at 65 °C by incubation with C1q alpha confirming the role of the complement.

In addition, to histamine and thrombin, other inflammatory mediators are able of inducing cell vesiculation. Starting from the observation that inflammatory cytokines modulate cell activation, Nomura et al. studied the effect of these molecules on the vesiculation of normal platelets under conditions of flow. These authors showed that IL-6, thrombopoietin and GM-CSF are inducers of platelet vesiculation in vitro.

In 2011, Brown et al. have shown that platelet activation in vitro by LPS (lipopolysaccharide) via TLR4 (toll like receptor4) could induce the production of proinflammatory platelet-derived MPs rich in IL1b. In a recent study, Soop et al. showed that the injection of low-dose LPS in healthy subjects induced increase PMPs.

Platelet aggregation induced by monoclonal antibodies may be accompanied by the formation of MPs. This action is independent of complement, and was observed with antibodies directed against the CD9, the receptor for the Fc fragment of immunoglobulins (FcyRII) or CD41 (GPIIb/IIIa). These observations led to involve antibodies in the mechanisms responsible for high levels of circulating platelet MPs in patients having an immune thrombocytopenia. The number of MPs released by platelets in the presence of serum from patients with heparin-induced thrombocytopenia is directly correlate with the presence of antibody anti-PF/heparin isotype IgG. Similar observations link platelet vesiculation, induced by patient serum having an ITP, to the presence of autoantibodies against platelet glycoproteins GPIIb / IIIa or GPIb. The role of antibodies is confirmed by animals models of immune thrombocytopenia, since the injection of anti-platelet antibodies induced in mouse thrombocytopenia associated with increased PMPs.
**Red blood cells**

Beside the calcium ionophore and the calcium chloride used *in vitro*, red blood cells produce vesicles during their maturation from reticulocytes to mature red blood cell, during their aging, and during storage or *in vivo* by budding.\(^{490}\)

It has been shown that approximately 20% of haemoglobin in circulating erythrocytes is loss during their lifetime, by this phenomenon of vesiculation.\(^{491}\)

The vesiculation during the red blood cell aging *in vivo* is associated with structural and functional changes of the protein band 3, anion exchanger and major protein of red blood cells membrane.\(^{492}\)

In addition, old studies report the formation of erythrocyte vesicles *in vitro* by depletion of glucose or ATP in the culture medium.\(^{493}\)\(^{494}\)

**Leukocytes**

MPs from leukocyte may be derived from neutrophils, monocytes or lymphocytes. Depending of the stimulus, Nomura et al. have reported that the stimulation of monocyte cell line U937 by inflammatory cytokines such as IFNγ (interferon gamma), IL2 (interleukin 2), IL-6 (interleukin 6) or GM-CSF (granulocyte growth factor and monocyte) induces the production of CD68 + MPs.\(^{495}\)

Furthermore, stimulation of monocytes by LPS causes the production of MPs able to disseminate a both pro-coagulant and pro-adhesive activity. The LPS is a ligand for toll-like receptor 3 (TLR3).

Gauley et al. showed that *in vitro* the LPS, but also the pathogen-associated molecular patterns (PAMPs) such as poly (I: C), another TLR3 ligand, induce MPs formation in a model of macrophages. This phenomenon depends on the nitric oxide (NO), which can itself induce vesiculation.\(^{496}\) Several recent studies have shown that administration of low-dose of LPS in healthy subjects caused an increase of monocytes circulating MPs. Aras and al. described this effect eight hours after injection LPS.\(^{496}\)\(^{497}\)

Granulocytes vesiculation was initially observed following the activation by complement molecules.

FMLP (N-formyl-methionyl-leucylphenylalanine) is often used to induce vesiculation of neutrophils or monocytes.\(^{498}\) Similar associations, PAF-PMA (Platelet activating factor/phorbol 12-myristate 13-acetate) or PHA (phytohemagglutininA)-PMA, induce an effective vesiculation on lymphocytes.\(^{499}\) In some experiments, T cells were activated with beads coupled to anti-CD3 antibody and anti-CD28.\(^{500}\)
**Endothelial cells**

EMP s are complex vesicular structures released by activated endothelial cells or apoptosis. Our group was the first to describe the generation of EMPs by HUVEC (human umbilical vein endothelial cells) stimulated by TNFα.

Other molecules can induce the generation in vitro of EMPs such as inflammatory cytokines, the LPS, reactive oxygen species (ROS), the inhibitor of plasminogen activator 1 (PAI-1), the thrombine, the camptothecin (an inhibitor of topoisomerase I), the C-reactive protein (CRP) and uremic toxins

Under certain conditions the endothelial NO synthase has a role in the production of MPs from endothelial cells.

The in vivo vesiculation drivers are less known; Boulanger et al. showed that the low shear forces are correlated with the rate of EMPss in patients with end stage renal disease, suggesting that the shear forces influence their production.

In recent years, several studies allowed to advanced our understanding on the comprehension of molecular mechanisms leading to the formation of EMPs. Curtis et al. showed that vesiculation of HUVEC induced by TNF-α depends on the p38MAP kinase way.

In our group, the work of C Sapet and S Simoncini showed that the activation by thrombin took place in 2 stages: an early stage, after binding to its receptor PAR-1 (protease-activated receptor-1) dependent on the activation of the Rho kinase ROCK-II by caspase 2 in the absence of cell death; a second phase later, dependent from transcriptional events associated with the TRAIL/TRAIL-R2 pathway. TRAIL / Apo2L is a cytokine that belongs to the family of TNF-α, secreted by the stimulated endothelial cell. TNF-α is required to produce EMPs during the early and late phase of the cells activated by thrombin. This soluble form of TRAIL binds to TRAIL-R2 receptor, recruiting proteins adapter to a death domain (TRADD) and TRAF2, RIP1-NFκB, participating in a loop amplification for the formation of EMPs. Stimulation of endothelial cells by the thrombin in HMEC-1 model, also led to an overexpression of IL-1Rα. The commitment of this receptor induces recruitment of protein adapters and TRAF6/Iraq1 which leads to an amplification of the phenomenon of vesiculation (Figure 20).
Figure 20: Molecular mechanisms controlling the formation of MEPs after stimulation of PAR-1 thrombin or after activation by TNF

The early stage of response to thrombin leads to the outsourcing of PS and to the activation of some caspases, Rho-kinase and the nuclear factor kappa B (NFkB) and to the expression of proinflammatory cytokines such as IL liberation of MPs.

(From Dignat George & Boulanger. ARVB. 2011)

2.3.4 Apoptosis

The apoptosis is a process during which cells induce their destruction in response to a signal. It is characterized by cellular shrinkage, DNA fragmentation and membrane budding behind the MPs vesiculation.

Some observations on THP-1 and U937 monocytic lines showed that there is a relationship between the MPs formation and the degree of apoptosis, evaluated by the proportion of hypodiploid cells, induced by oxysterols, the etoposide or the combination cycloheximide/actinomycin D. Similarly, cultured endothelial cells incubated in the presence of camptotecine, inhibitor of
topoisomerasis I, release MPs. The leukemia lines Jurkat T produces more effectively MPs after induction of apoptosis by treatment with staurosporine or analog 7-hydroxy (UCN-01) or with other inhibitors of PKC (protein kinase C) or CDK inhibitors (cyclin-dependent kinase). The relationship between apoptosis and vesiculation has been showed in vivo in human atherosclerotic plaques. Indeed, apoptotic cells (positive for TUNEL labelling) are localised in the plaque with PS positive procoagulant MPs expressing the tissue factor. The activation of caspase-3, the main effector of apoptosis, causes platelet vesiculation in animal models in vitro, and caspase inhibitors reduce the formation of PMPs because of TNF injection. Membrane budding depends on Rho-kinase I and II activation, activated during the process of apoptosis by its cleavage by caspase 3, itself activated by inducers of apoptosis (Figure 21). This suggests that MPs generated from cells undergoing apoptosis may contain nuclear materials; in this case the border becomes so slim that these vesicles can be considered apoptotic body.

Some studies describe a different molecular profile of MPs produced by apoptosis or cell activation. For example, Garcia et al. showed in cardiac transplant patients that EMPs circulating induced by apoptosis are richer in constituent markers of endothelium such as CD31 (PECAM: platelet endothelial cell adhesion molecule-1), compared to inducible markers such as CD54 or the CD62E increased only during the activation of the endothelium.

![Figure 21: General mechanism of MPs formation during apoptosis](From de VanWijk et al. Cardiovascular Rese arch.2003).
The vesiculation inductors acting on the multiple cellular types are coupled to different mechanisms of formation. This results in the generation of a large diversity of MPs in their composition. This structure and composition are completely related to functions panel carried by MPs.

In order to avoid confusion, it is important to underline that the so-called apoptotic bodies differ from the MPs, although apoptosis itself also elicits MPs release. Yet, the apoptotic bodies are released in the final stages of the programmed cell death and their size is larger than 1000 nm. In contrast, TMPs are formed at the beginning of the apoptosis with a size smaller than 1000 nm and do not contain organelles.

The mechanisms involved however are believed to be similar for most cell types. Cell activation leading to TMPs formation is attributed to various stimuli such as serine proteases, thrombin, ADP, inflammatory cytokines (e.g. TNF), growth factors, shear and stress inducers.

In contrast to this, apoptosis-induced TMPs release is regulated by the caspase-mediated Rho effector protein ‘Rho associated, coiled-coil containing protein kinase 1’ (ROCK 1), and can also be stimulated by TNF and thrombin.
2.4 CONCLUSIONS

Microparticles could represent future noninvasive biomarkers to define the etiology of pleural effusions. These extracellular vesicles, resulting from the blebbing of cell membrane in response to activation or apoptosis, are released by all eukaryotic cells, including cancer cells. Therefore, TMPs are currently studied as non-invasive biomarkers correlated with the diagnosis, the prognosis and the monitoring of cancer and with the response or the resistance to therapy. In particular, TMPs derived from pleural liquid could have an interesting role in differential diagnosis of pleuresis, distinguishing between malignant and benign pleural effusion. Moreover, they could have a role in the pathogenesis of the disease and they may represent a possible future target for biological therapy.

We will describe this topic in the next chapter.
CHAPTER 3: TUMOR DERIVED MICROPARTICLES

3.1 INTRODUCTION

3.1.1 Definition of tumor derived microparticles (TMPs)

The first observations of cancerous microparticles was documented in 1962, when tumor vesicles were clearly visible on murine teratocarcinoma images. In 1967, they were also described on induced renal tumor in the rat and they were called "vesicular structures" or "fragmented material". TMPs were also reported in 1978 when they were detected in cultures from a patient with Hodgkin disease. Another report, dating to 1985, is about small particles derived from melanoma cells. In the following decade, these vesicles were isolated from murine tumor cell lines supernatant, ascites fluid of patients suffering from ovarian cancer and villous adenoma (Fig. 22).

Figure 22: Cancer-derived Microparticles

(From Scott et al., J CellSci 1979)
Very quickly, the interest has focused on the relationship between these fragments and cancer metastasis. Tumor cells can produce TMPs constitutively without any apparent need for stimulus, but vesiculation can be increased by stress including exposure to chemotherapeutic drugs and heat. A series of papers reported TMPs detection in plasma, serum and other body fluids, as well as in supernatants of cells in culture.

The notion that TMPs are products of many – and possibly all – cells, and are actively shed in a finely regulated manner, has completely ruled out the possibility that they represent an artifact of purification. Tumor-derived microvesicles are heterogeneous membrane-bound sacs that are shed from the surfaces of tumor cells into the extracellular environment (Fig. 23).

Although different characteristics of these TMPs have been reported, all reflect the special potential of tumor cells for survival and for the expansion of the tumor, independently from cell-to-cell contact. They have been thought to deposit paracrine information and create paths of least resistance, as well as be taken up by cells in the tumor microenvironment to modulate the molecular makeup and behavior of recipient cells.

**Figure 23: Tumor-cell-derived microparticles**

*(From Tumor-cell-derived microvesicles, Martins et al.)*
Extracellular microvesicles (MPs) as ‘signaling platforms’. MPs are limited by a lipid bilayer membrane that contains integral membrane proteins such as MHC Class I and II, integrins, tetraspanins, and many others. These membrane proteins mediate interactions with specific ligands on target cells, initiating downstream signaling cascades in target cells. Membrane fusion between MPs and target cells results in the release of MP content (functional miRNA, mRNA, and proteins) into the cytoplasm, which can in turn modulate the gene-expression program of the target cell.

TMPs derived from cells represent specialized molecular and functional compartments, therefore, tumor cells may release different subtypes of TMPs\textsuperscript{531 532}. Current criteria to distinguish between different TMPs populations are based on size, density, subcellular origin, function and molecular cargo\textsuperscript{332}. TMPs concentration \textit{in vivo} increases as the disease progression. Their release is enhanced by activation or upon the initiation of apoptosis. TMPs are released from the surface of cells by the process of outward membrane budding through a loss of calcium-dependent membrane phospholipid asymmetry and cytoskeletal rearrangement\textsuperscript{405}. They are therefore composed of fragments of the parent cell, which comprise the plasma membrane proteins and cytoplasmic and nucleic constituents of the parent cell. Once TMPs bud from the parent cell, they are released into the systemic circulation, where they can effectively deliver their cargo long-range to recipient cells. In this way, TMPs serve as systemic vehicles in mediating intercellular communication. TMPs are fully equipped with all those factors (components) which enable tumor cells to escape immune surveillance; moreover, they facilitate the propagation of the tumor, preparing a “niche” for newly generated and dispersed tumor cells. Tumor cells emit more than one type of membrane vesicle, each with unique morphological traits and functions. Indeed, similar to hematopietic and vascular extracellular vesicles, the main subsets such as exosomes, microparticles/microvesicles and apoptotic bodies have been reported. However, a specific entities has been described called "oncosomes". 

\textbf{Oncosomes} represent an additional class of tumor-derived EVs, so called because of an atypically large size and abundant oncogenic cargo\textsuperscript{531 533}. Similarly to ectosomes, this MPs population might originate directly from plasma membrane budding and, like MPs, these particles express ARF\textsubscript{6}\textsuperscript{531}. Oncosome formation is particularly evident in highly migratory, aggressive tumor cells with an amoeboid phenotype, and experiments in different cell lines
indicate that oncosome can form as bioproducts of non-apoptotic membrane blebs used by amoeboid cells as propulsive forces to migrate\textsuperscript{531, 533, 534}. Oncosome have been identified in several cancer systems, and comparative experiments between tumor and benign cells indicate that they are specifically released by tumor cells, whereas their detection in benign cell systems is negligible. Consistently, oncosome-like features have been described in human prostate cancer sections \textit{in situ}, but not detected in benign tissue\textsuperscript{531}. Oncosome shedding is common to several tumor types, including prostate, breast, bladder, lung cancer, and other tumors (\textsuperscript{531} and unpublished observations) and is enhanced by silencing of the gene encoding the cytoskeletal regulator Diaphanous related formin-3 (DIAPH3), or by activation of the epidermal growth factor receptor (EGFR) and overexpression of a membrane-targeted, constitutively active form of Akt1. Importantly, oncosome have been identified in the circulation of mice and patients with metastatic prostate cancer, suggesting that these MPs are potentially useful sources of clinical biomarkers\textsuperscript{535}.

MPs in the size range and appearance of oncosomes have been recently described with different names including: (1) giant vesicles, identified in ERα-positive breast cancer cells and tumor tissues\textsuperscript{536}; (2) migrasomes, large round structures containing numerous vesicles (pomegranate-like structures), which depart from retraction fibers of migratory benign cells\textsuperscript{537}; and (3) tumor-derived MPs originating from amoeboid-like tumor cells, in which VAMP3 seems to regulate the delivery of MPs cargo to regions of high plasma membrane blebbing. MPs appear to be released through blebbing during migration\textsuperscript{538}. Whether these three types of MPs are distinct from oncosomes and use different mechanisms to play their extracellular functions is currently unknown.

Despite the effort to reach a consensus on vesicle nomenclature and classification, it is becoming evident that the biochemical composition and the biological function of different MPs populations derived from the same cellular system overlap, at least in part\textsuperscript{539}. Furthermore, current methods of isolation do not discriminate between exosomes and ectosomes because physical properties that can unambiguously distinguish between the two MPs types have not been fully characterized and specific molecular markers are still lacking\textsuperscript{463, 540, 541}. However, despite the current limitations, a recent study indicates that RNA profiles of exosomes, ectosomes, and ABs differ\textsuperscript{542}. Therefore, regardless of the confusing and frequently inappropriate terminology used to define particular MP populations, it is clear that tumor cells release a spectrum of MPs that might all functionally participate in the biology of the disease. A more comprehensive introduction into EVs, particularly exosomes, is provided in a focus edition by Kalra \textit{et al.}\textsuperscript{543}. 
3.1.2 Biogenesis and formation of TMPs

Given emerging literature documenting the unexpected ways TMPs can influence disease progression, understanding the mechanisms involved in TMPs biogenesis and shedding at the tumor cell surface is important, as it could provide novel and key cancer therapeutic strategies. Fission of TMVs from the cell surface to allow “shedding” is facilitated by actin–myosin-based contraction via a process that also appears to involve the ADP-ribosylation factor 6 (ARF6) protein. ARF6-mediated ERK (extracellular signal–regulated kinases) activation seems to have a regulatory role in facilitating localized activation of myosin light chain kinase and, subsequently, the phosphorylation of myosin light chain to promote the release of TMVs from invasive melanoma cells. This ERK induction required ARF6-induced activation of phospholipase D (PLD). A study examining PS externalization in platelets showed that phosphatidylinositol 4,5-bisphosphate (PIP2) is necessary for efficient PS externalization, providing a potential link to ARF6-regulated PIP2 generation and PS externalization. Of note, blocking ARF6 activation in a melanoma cell line drastically reduced TMV release, and ARF6 is detected on TMVs released from a spectrum of tumor cell lines, suggesting that common pathways dictate TMV biogenesis. MHC class I, β1-integrin receptors, and VATMP3—all of which trafficked to the plasma membrane via ARF6-regulated endosomal recycling—were present in nascent as well as shed TMPs. Intriguingly, however, transferrin receptors, which are abundant at the cell surface and trafficked on ARF6 endosomes in some cell types, were not recruited to TMPs. In addition, cortactin and Tks5, components of invadopodia, which are membrane protrusions formed at the adherent and invasive front of the tumor cell, were also absent from shed TMPs. In addition to the cargo mentioned above, the ARF6-positive TMPs also contain MT1-MTTP, which has been thought to traffic along the early and late endocytic recycling pathways. Collectively, these findings suggest that in tumor cells, specialized ARF6-positive recycling vesicles target protein cargo to the cell surface for incorporation into TMPs.

The demonstration that TMV shedding is linked to suppression of Diaphanous-related formin-3 (DRF3) expression provides further evidence for the involvement of actin cytoskeleton-based fission. DRF3 is a signaling protein that binds small Rho family GTPases and has been implicated in actin nucleation. Loss of DRF3 promotes cellular changes conducive to the formation of microvesicles and the acquisition of the amoeboid phenotype.
Intriguingly, in this regard, more recent work has implicated RhoA signaling in the generation of TMVs with downstream activation of ROCK (Rho-associated coiled-coiled-containing protein kinase) and LIM kinase downstream from RhoA activation. Li et al. recently identified RhoA, a member of the small GTPases family, together with its downstream targets Rho-associated coiled coil containing protein kinase (ROCK) and the LIM kinase (LIMK) as a regulator of TMPs release. Whether RhoC, whose activated form is similar to RhoA, is involved in the process is still a matter of debate.

Crosstalk between the ARF6 and Rho signaling pathways is essential to the coordination of TMV release. Also interesting to note are the commonalities between mechanisms and signaling pathways that govern TMV formation and membrane blebbing at the cell surface. Moreover, lipid raft domains seem to be abundant in TMPs, and TMP formation can be impaired by cholesterol depletion. In fact, TMPs biogenesis and shedding occur at regions on the cell membrane that appear to be enriched in specific lipids. One of these required lipids is cholesterol; pharmacological depletion of cellular cholesterol inhibits microvesicle release.

In addition to rearrangements in the plasma membrane composition, proteins responsible for cell shape maintenance may be involved in TMPs biogenesis, by regulating actin dynamics. Receptor tyrosine kinases such as EGFRvIII, which are also present in TMPs, may also be transported intracellularly to TMPs via early endocytic recycling, although this has yet to be demonstrated.

More recent studies have shown that caveolin-1 is targeted to MVBs and lysosomes, leading one to speculate that membranes derived from these compartments may also direct cargo to TMPs. Thus, while the ARF6-regulated recycling pathway is one mechanism to direct cargo to sites of TMPs biogenesis, nascent microvesicles at the cell surface may be a convergence point for multiple membrane trafficking pathways directing specialized cargo to these structures.

As indicated above, genetic material—including mRNAs, microRNAs (miRNAs), genomic DNA, and retrotransposons—is also found in microvesicles derived from a wide spectrum of tumor cell lines, including glioblastoma, colon, and gastric cancers. Here again, due to the heterogeneity of both TMPs populations and isolation techniques, one cannot exclude the possible contribution of several vesicle populations to the studies described. It is unclear how nucleic acids are targeted to the tumor cell surface or specific intracellular compartments. That there is enrichment of certain nucleic acids in microvesicles suggests that similar to protein cargo, nucleic acids may also be selectively packaged into microvesicles. While it is possible...
that cytoplasmic nucleic acids might traffic to sites of microvesicle biogenesis as complexes along with lipids or proteins on cytoskeletal track\(^\text{558}\), how genomic DNA might be incorporated is less clear. One possibility is that amplified genomic sequences escape into the cytoplasm during mitosis following nuclear envelope breakdown and are then trafficked as complexes to specific sites in cells for packaging into TMPs.

A subgroup of budding microvesicles is driven by interaction between Tsg101 and ARRDC1 (arrestin domain-containing protein 1) proteins, resulting in the relocation of Tsg101 from endosomes to plasma membrane and the release of microvesicles containing Tsg101, ARRDC1, and other cellular proteins\(^\text{525}\). Unlike exosomes, which are derived from multivesicles body (MVBs), ARRDC1-mediated microvesicles (ARMMs) lack late endosomal markers.

Unraveling the mechanism of TMPs biogenesis is a biologically relevant goal that might shed light on extracellular communication and result in clinically applicable tools, including development of new therapies.

The sorting of TMPs cargo seems to occur during TMPs formation, suggesting that the two processes might be interconnected, and molecules exported in MPs might also be functionally involved in their biogenesis.

### 3.1.3 Cargo of TMPs

Initially identified as a mechanism by which cells could dispose of damaged or unnecessary proteins\(^\text{559}\), cell-derived microvesicles are now seen as signaling particles that contain lipids, proteins, and nucleic acids which carry molecular information both locally and at distant sites, shaping cell microenvironment during development and cancer progression\(^\text{557 560 561 562 563}\).

Microvesicles recovered from cells or from body fluids are very heterogeneous in composition\(^\text{560}\).

Both the microvesicular membrane and its internal content include general and specific components. The general ones are common for practically all TMPs, these are membrane lipids, membrane proteins, cytoskeletal proteins and some chaperone proteins.

The membrane phospholipid asymmetry is different in TMPs from the intact cell. Before budding the negatively charged phosphatidylserine (PS) and phosphatidylethanolamine (PE) accumulate in the outer layer, due to an increased intracellular \(\text{Ca}^{2+}\) level, which inhibits the original asymmetry-maintaining action of the floppase, translocase and scramblase enzymes.

Annexin V binds to MVs thanks to the nonstandard membrane asymmetry and, consequently,
macrophages are also attracted\textsuperscript{337,343}. In accordance with their origin, TMPs contain endocytic markers, i.e. tetraspannins and hsp73\textsuperscript{336,526}.

Besides the commonly displayed molecules, the specific protein composition of the TMPs reflects the nature of the donor cell, in as much as their cell surface proteins, their membrane fusion and receptor proteins and their adhesion proteins are equal to those of the parental cell. Because TMPs may contain miRNA they can potentially effect epigenetic changes in recipient cells, meanwhile the mRNA molecules represent a new translational potential. DNA fragments have also been identified in MVs\textsuperscript{564,565}.

Besides the proteins which are ubiquitous among TMPs, these microvesicles serve as vehicles for several tumor-specific determinants, such as Her-2/neu and mesothelin. They carry tumor antigens such as MelanA/Mart-1, CEA, HER-2 and the MHC I and MHC II (HLA-G, HLA-E) complexes for antigen presentation. They carry apoptosis-inducing TNF, FasL and/or TRAIL and immunosuppressive TGF. They were shown to contain mRNA for -actin and adhesion proteins, for chemokine IL-8, for VEGF, HGF and for CD44, the cell surface receptor for hyaluronate.

In addition, drug transporters have been identified in their membrane\textsuperscript{336,566,567}.

The TMP membrane has a lower content of phospholipid than does the cell membrane, but is enriched for cholesterol and sphingomyelin\textsuperscript{568}.

This observation is noteworthy, because the membranes of highly metastatic cancer cells also have a greater fraction of spingomyelin than do those of less metastatic cells\textsuperscript{569}. This finding suggests that the tumorigenicity of TMPs is partly due to the abundance of sphingomyelin in their membranes. Moreover, sphingomyelin is required for angiogenesis. It plays an active role in inducing endothelial cell migration, as prerequisite for neovascularization\textsuperscript{570}.

The large fraction of PS in the outer phospholipid layer of the TMPs membrane also contributes to shaping the tumors’ microenvironment. Because they attract macrophages, PS molecules draw macrophages to the site\textsuperscript{337,343}, they can induce inflammation, a beneficial milieu for tumors. Interestingly, exosomes are enriched in ceramides. This increased ceramide content of the membrane seems to be necessary for the sorting of vesicles into different TMPs at the very early stages of the endosomal–lysosomal pathway: to decide whether the endosomal content will be degraded in lysosomes or to select those vesicles, which will be released as TMPs\textsuperscript{571}.

**Proteins**

Oncogenic receptors form an important group of bioactive cargoes packaged in TMPs that can significantly modulate the microenvironment. For example, when a nonaggressive population
of tumor cells was exposed to EGFRvIII protein obtained from aggressive glioma TMPs, mitogen-activated protein kinase (MAPK) and Akt were induced, leading to morphological transformation of these recipient cells and an increase in anchorage-independent growth. Notably, treatment of the TMVs with EGFRvIII kinase inhibitor or masking their exposed PS residues with annexin V diminished the downstream effects in the recipient cells, further suggesting a link between the downstream effects in the recipient cells and the acquisition of EGFRvIII from TMVs. Another example is the AXL receptor in TMPs shed from chronic lymphoblastic leukemia (CLL). AXL-positive TMPs conditioned the bone marrow to enhance CLL disease progression.

TMPs are also loaded with proteases, which provide an additional means of matrix degradation, and likely promote focal proteolysis at more distant sites to create a path of least resistance for metastatic cells. Accordingly, MTMP-2, MTMP-9, MT1-MTMP, and their zymogens urokinase-type plasminogen activator (uPA) and EMTMPRIN have been discovered within TMPs.

It has been proposed that β1-integrins facilitate the interaction of shed vesicles with the ECM and that the characteristically acidic pH of the tumor environment promotes microvesicle burst and content release. This mode of matrix degradation might be especially important, as tumor cells traverse long distances and assume the amoeboid phenotype that is characterized by high levels of non-apoptotic blebbing, reduced protease activities, and a deformable plasma membrane.

The amoeboid phenotype is distinct from the “mesenchymal” phenotype, in which the cells are elongated and fibroblast-like. Amoeboid movement through tissue spaces can be rapid and only minimally dependent on repetitive cycles of membrane attachment to ECM and retraction. TMPs shedding from amoeboid tumor cells could condition the extracellular milieu through deposition of proteases and other paracrine signals to allow the cell to traverse and invade at long distances. This hypothesis would also be consistent with findings that support an absolute dependency on protease-mediated degradation of tumor invasion through ECM. TMPs are also thought to carry cargo to modulate the immune response of the tumor microenvironment. This aspect of TMP function has been extensively reviewed and is an important feature of disease progression.

Examples of how TMPs aid in evasion of the immune response are as follows: Direct fusion of microvesicles produced by human melanoma or colorectal carcinoma cells with monocytes inhibits monocyte differentiation to antigen-presenting cells but promotes release of immunosuppressive cytokines, inhibited cytolytic T-cell activation, and function.
reports have shown that TMPs with exposed FasL, a ligand of the death receptor Fas (CD95), will induce apoptosis in activated T cells and thereby abrogate the potential of these effectors to kill tumor cells

Furthermore, TMPs shed by lymphoblastoma cells contain latent membrane protein (LTMP-I), another immune-suppressing transmembrane protein, which inhibits leukocyte proliferation. TMPs are also thought to harbor proangiogenic regulators, including VEGF and bFGF. Angiogenesis is a hallmark feature of tumor growth and survival and is characterized by endothelial cell proliferation to form blood vessels that infiltrate into the tumor.

Al-Nedawi et al. (2009) showed that the transfer of EGFR from TMPs shed by human cancer cell lines harboring the activated EGFR mutation results in the onset of VEGF and VEGF receptor expression in endothelial cells.

TMPs shed from ovarian cancer cells have also been shown to promote the angiogenic capabilities of endothelial cells via a mechanism that requires CD147 (also known as extracellular matrix metalloproteinase inducer) found on shed TMPs.

In addition, reports indicate that proteins present on TMPs stimulate secretion of several proangiogenic factors by stromal fibroblasts to facilitate angiogenesis via the proliferation of endothelial cells.

Microvesicles shed from endothelial cells, monocytes, and platelets harboring similar cargo would only help to amplify such a coagulation response.

**Nucleic acids**

Nucleic acids packaged in TMPs include mRNAs, miRNAs, noncoding RNAs (ncRNAs), and cDNAs.

The presence of nucleic acids, specifically mRNA, was first reported in shed vesicles released from stem cells in 2006 and then 1 year later from mast cells. These compelling and unexpected discoveries led to the fascinating idea that perhaps shed microvesicles, like cell-extruded viruses, can transfer genetic information between cells.

As described here, and previously for protein cargo, this type of intercellular transfer of genetic information was shown to have a marked effect on the tumor microenvironment.

In a classic example, TMPs shed from glioblastomas contain mRNAs that promote tumor growth, invasion, and immune repression.

These TMP mRNAs are taken up by brain endothelial cells in culture to promote angiogenesis. TMPs containing mRNAs have also been reported for colon and gastric cancers. Interestingly, there appears to have overlap in transcripts contained in TMPs from these different cell types, suggesting that TMPs, independent of tissue source, likely have common
influences on the surrounding microenvironment. MiRNA have also been described in TMPs shed from several tumor cell lines, including lung, glioblastomas, and gastric cancers. Through the negative regulation of multiple mRNA targets in recipient cells, miRNAs have the potential to markedly affect the tumor microenvironment. However, the mechanisms by which miRNAs in TMPs might facilitate changes to the transcriptome of target cells are far from understood. More recently, TMPs released from CD105-positive human renal cancer stem cells were shown to stimulate angiogenesis and the formation of a lung premetastatic niche. Molecular characterization of these CD105-positive TMVs included a set of proangiogenic mRNAs and miRNAs implicated in tumor progression and metastases.

Retrotransposons, cDNAs, and ncRNAs have also been reported to be present in TMPs. It is intriguing that a large component of nucleic acids packaged into TMPs is ncRNAs, although their role in disease progression awaits further research. Some ncRNA cargoes have been implicated in cell function and gene regulation at both the transcriptional and post-transcriptional levels. Retrotransposon RNAs such as LINE-1, HERV-K, and Alu have also been detected in TMPs. The same study showed that when HERV-K-rich TMPs derived from human medulloblastoma tumor cells were exposed to human endothelial cells, the HERV-K sequences in the recipient endothelial cells were significantly elevated. By inserting themselves into the genome, these retrotransposon elements have the capacity to modulate and manipulate genomic content. Although normally silent, during cancer, they may be activated and result in an increased number of these repetitive elements, which enhances genome plasticity. Thus, the most direct and potentially significant consequence of TMP release is their ability to modulate the behaviors of stromal cell populations. Once integrated, the consequences to target cell function are now recognized, but the exact fate of the cargo in recipient cells requires further investigation.

MVs can act as a “signalling complex” as well as a “recipient cell-modifying complex” through surface-expressed ligands, through the transfer of membrane proteins (receptors or receptor coupled proteins), through the delivery of cytosol proteins, lipids, siRNA, miRNA and even pathogens. They provide a more stable conformational condition to the protein content, since the milieu of the proteins does not get altered, like it does when the proteins are simply secreted into the extracellular matrix. Thereby the bioactivity of the proteins will be also increased in a transmembrane form. Finally, MVs improve the biological distribution of molecules, since they
act in a paracrine manner and as remote messengers. These attributes contribute to create a highly efficient information flow between cells through the “microvesicular network”.

3.1.4 Detection and Isolation of TMPs

TMPs can be isolated from conditioned media of cultured cells as well as from virtually any type of body fluid, including blood and derivatives, urine, ascites, bronchoalveolar lavage (BAL), saliva, and cerebrospinal fluid (CSF). The attempts to perfect current methods of TMPs isolation have recently lead to increased understanding of the biological function and nature of diverse types of TMPs. Along with much progress, unexpected complexity has emerged. One of the most commonly employed methods of TMPs isolation is based on differential centrifugation. Discontinuous cushion gradients represent a viable alternative to continuous gradients that can be cumbersome. With these methodologies, TMPs can be purified through flotation of the different TMPs populations at known concentrations of sucrose, iodixanol or other agents. Recent studies have also highlighted that differential centrifugation, known to result in heterogeneous preparations, is not sufficient to isolate pure populations of TMPs. TMPs can be better purified and cleared from free proteins and protein complexes or other contaminants by centrifugation gradients, typically used to separate different intracellular organelles based on their sedimentation coefficient. It is to be mentioned, that the purification methods represent the most critical and crucial step in the microvesicular studies. Treatments with TMPs should exclude the presence of all other molecules, which circulate in blood or are secreted into the supernatants, although washing and purifying techniques cannot fully ensure pure TMPs pool, without damaging the integrity of the microvesicular membrane. Also larger vesicles can disperse to smaller ones, or smaller ones can fuse in response to mechanical forces. This implicates that the vesicular size on the electron microscopic scale should be only an indication regarding the type of the vesicle. Size exclusion methods based on the use of filters with specific pore size are often used in combination with other isolation techniques. This is frequently the first step in exosome purification from serum and plasma. In conventional protocols, after pelleting down cells and cell debris at low speed, TMPs are purified at 10,000-20,000 × g, as is the case for TMPs and oncosomes. Error! Bookmark not defined. or at 100,000-120,000 × g as for
exosomes. However, different variations of this protocol have been employed, resulting in confusing results. Some authors recently developed a size retention rather than exclusion isolation method able to select TMPs larger than 200 nm, which include large oncosomes, while allowing smaller TMPs to flow through the filter. Using this method, large oncosomes positive for Cav-1 were shown to discriminate patients with locally confined prostate cancer from patients with castration resistant and metastatic disease.

*Immunoaffinity capture* is emerging as a new tool to purify specific TMP populations based on the expression of certain membrane proteins. For example, microbeads coated with glycoprotein A33 and epithelial cell adhesion molecule precursor (EpCAM) have been successfully used to immunocapture different TMPs from colon cancer cells. Novel and promising methodologies include microfluidic systems that allow TMPs immunocapture using specific antibodies.

One elegant system, with high potential for clinical applicability, has been used to quantitatively analyze TMPs in the serum of patients with glioblastoma using general markers and EGFRvIII. Because of the increasing technical ability to stratify heterogeneous TMPs populations, several markers that have been considered exosome-specific, including CD63, CD81, CD9 are now being identified in other types of TMPs, whereas proteins such as Alix and Tsg101 appear to be more consistent markers. PS, ARF6 and Rho family members have been proposed as TMPs markers. However, cross-reactivity with other TMPs has been demonstrated, limiting the current understanding of potentially different functions and clinical significance for these two classes of TMPs. Whether large oncosomes represent a discrete population of TMPs is still unresolved, and whether TMPs and large oncosomes express different markers has not yet been explored. Identification of sets of markers rather than single ones by multiplexing techniques and large scale mass spectrometry, including targeted proteomics will improve our ability to purify TMPs species with different signatures *in vitro* and *in vivo*. If performed under well-controlled conditions, these experiments, followed by extensive validation, can improve the sensitivity and specificity of currently available methods.

A range of *imaging methodologies* has been applied to TMPs, contributing to the conclusion that TMPs are discrete, particulate structures with a lipid bilayer. Nano-sized TMPs and MVs require the resolution power of electron microscopy (EM) to be visualized. Larger TMPs, such as large oncosomes, can be visualized by confocal or optical microscopy in tissue plasma membranes, and measured using imaging software. Immuno-fluorescence imaging allows identification of large oncosomes also in cell media and body fluids. Notably, in formalin-
fixed, paraffin embedded (FFPE) sections, large oncosomes can be highlighted by chromogenic immunohistochemistry, supporting a high potential for clinical translation in cancer. **Flow cytometers** are capable of sorting and capturing microvesicles based on both physical characteristics (size) and affinity (using fluorescently conjugated antibodies). To determine distributions of vesicles, cytometers make use of a combination of both forward and side-scatter measurements. In this technique, researchers often used a series of polystyrene beads with defined sizes to calibrate the equipment and then gate the experimental population at or just below 1-mm-diameter beads. However, this analysis is limited to vesicle populations that are >200–300 nm in diameter due to constraints imposed by the wavelength of the laser. A recent study from van der Vlist demonstrated that nano-sized particles can be accurately quantified. Interestingly, using a multicolor labeling strategy, this approach could be used to stratify subsets of heterogeneous TMPs. Recent studies further demonstrate that nano-sized TMPs can be enumerated by flow cytometry with the support of antibody-coated beads larger than exosomes. Given their atypically large size, large oncosomes can be quantified in cell secretions, mouse and human plasma, with and without staining with fluorescently-labeled antibodies, using 1 and 10 μm size beads.

More frequently used for the study of nano-sized TMPs, the **nanoparticle tracking analysis** (NTA) system the light scattered by the particles can be captured and analyzed by computer software, resulting in a measurement of the size distribution and concentration of the TMPs in the samples. This method is not suitable for quantitative analysis of TMPs larger than 400 nm.

**Newer systems**, based on dynamic as well as electrophoretic and static light scattering in combination, seem to allow quantitative analysis of TMPs of several microns in diameter. However, their application has been limited to the use of liposomes in drug delivery experiments.

As described above, the clinical promise of TMPs prompts the development of platforms to enable the isolation of low-abundance microvesicles in body fluids. TMPs comprise a relatively small fraction of the bulk fluids from which they can be isolated. Hence, a growing number of enrichment and isolation techniques have been aimed at efforts to effectively concentrate and capture these structures in a minimally invasive fashion for use as diagnostic or prognostic indicators. Many of these techniques, developed for the isolation of shed vesicle populations from a myriad of sources, show renewed promise for use in isolating and characterizing TMPs (Table 8).
<table>
<thead>
<tr>
<th>Methodology</th>
<th>Resolution</th>
<th>Fractionation and capture</th>
<th>Requirement/Assumptions</th>
<th>Time scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometry</td>
<td>~1 μm</td>
<td>Yes</td>
<td>Large fluid volume, no centrifugation</td>
<td>Hours</td>
</tr>
<tr>
<td>Dynamic light scattering</td>
<td>~50 nm-2 μm</td>
<td>No</td>
<td>Temperature and viscosity, mass of sample</td>
<td>Minutes to hours</td>
</tr>
<tr>
<td>Nanoparticle tracking</td>
<td>~200 nm</td>
<td>No</td>
<td>Sample preparation, flow rate</td>
<td>Minutes</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>&lt; 100 nm</td>
<td>No</td>
<td>Sample preparation, suspension in TEM buffer</td>
<td>Hours</td>
</tr>
<tr>
<td>Flourescence microscopy</td>
<td>&lt; 100 nm</td>
<td>No</td>
<td>Sample preparation, suspension in TEM buffer</td>
<td>Hours</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methodology</th>
<th>Resolution</th>
<th>Fractionation and capture</th>
<th>Requirement/Assumptions</th>
<th>Time scale</th>
</tr>
</thead>
<tbody>
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<td>Microfluidic sorting</td>
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<td>Large fluid volume, no centrifugation</td>
<td>Minutes</td>
</tr>
<tr>
<td>Optical trapping</td>
<td>~10 μm</td>
<td>No</td>
<td>Sample preparation, suspension in TEM buffer</td>
<td>Minutes</td>
</tr>
<tr>
<td>Magnetophoretic sorting</td>
<td>~20 μm</td>
<td>Yes</td>
<td>Large fluid volume, no centrifugation</td>
<td>Minutes</td>
</tr>
</tbody>
</table>

Table 8: Techniques for characterization, separation, and isolation of shed vesicle populations
(From D’Souza-Schorey and Clancy GENES & DEVELOPMENT, 2012)

### 3.2. BIOLOGICAL ROLE OF TMPs
TMPs have demonstrated to have almost all features of other most common MPs. Anyway, in this section, we detail the main roles of the microparticles derived from tumor cells and the highlights properties related to tumorigenesis (Fig.24).

Figure 24: Overview of the role of exosomes and microvesicles in promoting metastasis. The complex interplay between MVs derived from tumor (TCMVs) and from cells in the microenvironment favoring migration, invasion, and establishment of metastasis. TCMVs derived from tumor and cancer stem cells, together with MVs derived from cells of the tumor stroma, may prime the metastatic niche in lymphnodes and may facilitate vascular dissemination and extravasation of cancer cells at distant metastatic sites. TCMVs can also recruit and reprogram bone marrow progenitors toward a prometastatic phenotype.

MVs, microvesicles.

(From Tumor-cell-derived microvesicles Martins et al.)

3.2.1 Intracellular communication

The coexistence of many cell types within the same organism requires a high level of coordination, which is mediated by molecular mechanisms of intercellular communication.
Historically, soluble factors have been considered the central players in this process\textsuperscript{621, 622}. Soluble factors include secreted ligands that can bind plasma membrane receptors, thus activating signaling cascades in target cells\textsuperscript{623} (Fig. 25).

**Figure 25: Large oncosomes as new players in intercellular communication**

Tumor cells communicate with various components of the tumor microenvironment by EVs. While some of the most common mechanisms of interaction between tumor-derived MV (red dots) and exosomes (green dots) with target cells have been described, those that govern the cross talk between large oncosomes and the microenvironment are still largely unknown.

(From Minciacchi et al.)

Depending on the distance between originating cell and target cell, the principal categories of intercellular communication are: autocrine, in which the target cell and the secreting cell are the same; paracrine, in which the target cell is near the secreting one; and endocrine, in which the target is distant and the secreted factors travel great distances through the blood\textsuperscript{624}.

Cell communication can also be achieved by cell-to-cell contacts, as is the case for juxtacrine interactions\textsuperscript{624, 625, 626}.

Intercellular communication is essential for embryonic development as well as the functional integrity of multicellular organisms\textsuperscript{523, 627}.

Indeed, shed membrane vesicles serve to shuttle bioactive molecules between cells, and this cargo can modulate the extracellular environment\textsuperscript{315, 525}. 
In addition to depositing paracrine information, these membrane vesicles can also fuse with cells in the microenvironment to alter recipient cell content and behavior. The latter may result from direct membrane fusion or the endocytosis of the extracellular vesicle into the target cell. Since then, by their ability to harness select bioactive molecules and propagate the horizontal transfer of their cargo, shed microvesicles have been shown to have an enormous impact on tumor growth, survival, and spread, along with demonstrated effects on many stages of tumor progression, including angiogenesis, escape from immune surveillance, extracellular matrix (ECM) degradation, and metastasis. As TMPs are detached from cells, both their cytoplasmic fraction and their membrane contain a variable spectrum of molecules, whose pattern reflects specifically the donor cell which secretes them. The TMPs reach other cells, not only locally, but over long distances as well.

In the “classical” intercellular communication, cells secrete molecules serving as signals for the target cells. The ligand–receptor interaction initiates a signal transduction pathway within the cell, ultimately effecting changes in cellular physiology, such as metabolism, mitosis and apoptosis. Signalling by TMPs is qualitatively different: complex “packets” of information are transmitted to the target cell, since membrane domains and cytoplasmic components travel in the MVs. The vesicular content, delivered to the target cell, displays a wide range of molecules including proteins such as signal proteins and receptors as well just as much as cytoskeletal or effector proteins, lipids, DNA and RNA. Furthermore, prions and viruses have also been shown to infect the host cell through microvesicles. Depending on the microvesicular content, more stimuli and more information will be delivered to the cells simultaneously, allowing a more complex and more effective cellular response. Therefore, after having received new sets of proteins, the nature of the target cell may be altered, resulting in new responses, in new signalization pathways and further MV release can be elicited.

The interaction of TMPs with their target cells is not only mediated by membrane-membrane contact, but often results in TMP uptake with subsequent transfer of TMP cargo. Both fusion and active endocytosis have been proposed as mechanisms for exosome uptake. Exosome and MV binding and internalization can be regulated by adhesion molecules. For example, the interaction between diverse combinations of tetraspannin complexes, highly
represented on exosome membranes, and integrins on the target cell, might influence the 
selection of the recipient cell.\textsuperscript{632} TMP-cell interactions can also be mediated by the PS expressed on TMPs and TIM4 or other 
PS-specific receptors on target cells. Finally, a relevant role in TMP internalization seems to 
be played by heparin sulfate proteoglycans residing on the plasma membrane of the target cell, 
as recently reported by Christianson.\textsuperscript{633,634} Whether these processes are truly selective or rather can occur randomly is still largely unclear. 
The TMP-target cell interaction is the first step of TMP uptake, and seems to be unavoidably 
followed by fusion or endocytosis.\textsuperscript{635} Tumor derived TMPs can alter the homeostasis of the tumor microenvironment by directly 
targeting fibroblasts, endothelial and immune cells or by altering the structure and 
coTMPosition of the extracellular matrix (ECM).\textsuperscript{636,637,638,639,640} Along with several studies 
showing an TMP-mediated horizontal propagation of tumor promoting molecules, it is now 
emerging that TMPs can also spread acquired phenotypes and functions including drug 
resistance.\textsuperscript{641-642} A few recent studies have demonstrated that TMPs may mediate the transfer of nucleic acids, 
including mutated genetic material, a phenomenon that was previously thought to happen only 
vertically within clonal cells. The hypothesis that this might occur through direct transfer of DNA is attracting strong interest 
and has resulted in a few reports on the functional role of mutated DNA in recipient cells.\textsuperscript{643} Additionally, evidence is slowly emerging that TMP-enclosed DNAs can be transferred into 
cell nuclei, as demonstrated by acridine orange or lipophilic dye-labeled TMPs, identified 
inside the nuclear membrane.\textsuperscript{644} Whether the transferred DNA can exert a functional role on target cells is still unresolved. 
Oncogene transcripts can be propagated through TMP transfer and then translated into proteins 
in the recipient cells.\textsuperscript{594} Analogously, TMP enclosed miRNAs can regulate gene expression, thus altering the behavior 
of the recipient cells and increasing metastatic potential in poorly metastatic cells. An example is provided by recent findings on miR-200, in breast cancer metastasis, using a 
series of xenograft models.\textsuperscript{644,645} TMPs mediate the exchange of intricate intercellular messages comprised of classical soluble 
and insoluble signaling factors, as well as molecules of a different nature, including structural 
proteins, nucleic acids, and lipids. Additionally, TMPs can travel through body fluids, thus conveying functional information to
distant sites in vivo\textsuperscript{646}.
These and other findings have completely changed the concept of the nature intercellular communication, and have helped to clarify diverse cellular processes in pathological and physiological conditions (Table 9).

**Figure 26: TMVs serve for information transfer and for paracrine/endocrine signalling.**

TMVs, containing numerous biologically active proteins and RNAs dramatically change the phenotype of the recipient cell. They not only transfer information, but can reprogramme the recipient cells with the goal of favoring tumorigenesis.

(From E. Pap et al. / Critical Reviews in Oncology/Hematology 79 (2011) 213–223)

Microvesicle–target cell interaction results in either the fusion of the microvesicle with the target cell or endocytosis of the microvesicle\textsuperscript{647 648 649 650}. 
Table 9: Intracellular communication mediated by tumor-derived microvesicles (TCMVs)
(From Tumor-cell-derived microvesicles Martins et al.)

3.2.2 Modulation of the tumor microenvironment

TMVs are also thought to carry cargo to modulate the immune response of the tumor microenvironment. This aspect of TMV function has been extensively reviewed and is an important feature of disease progression. Once shed, TMPs enable the horizontal transfer of bioactive content and can thus have a profound influence on the tumor microenvironment. In addition to depositing paracrine signals, microvesicles have been shown to interact with cells in the tumor microenvironment, promoting signaling responses in the target cells.

Accruing literature suggests that shed TMVs can condition the stromal microenvironment to
promote angiogenesis, evasion of the immune response, tumor invasion, and, potentially, metastasis. TMVs released from tumor cells can be taken up by cells in the tumor microenvironment, such as carcinoma-associated fibroblasts, with consequences for target cell behavior (Fig. 27).

**Figure 27: TMV-mediated modulation of the tumor microenvironment.**

Tumor cells (brown), Carcinoma-associated fibroblasts (blue)

(From D’Souza-Schorey and Clancy)

They can also interact with the extracellular matrix by depositing paracrine information or facilitating matrix degradation, thereby creating paths of least resistance.

### 3.2.3 Procoagulant activity of TMPs

The TMPs have also been described as bearing a procoagulant activity dependent from Tissue Factor (TF). In fact, the expression of TF by tumor MPs is well described in the literature. Thus, the problem of the link between MPs and thrombosis associated with cancer is an important topic in the literature.

Davila et al have shown that injection of FT + MPs derived from cancer cells in mice causes the disseminated intravascular coagulation syndrome in vivo. Interestingly, this study also
shows that an injection of a low concentration of TMPs is necessary to overcome the anti-thrombotic protective mechanisms. These findings are consistent with the group C. Dubois show that injection of TMPs derived from human and murine pancreatic tumor cell lines induces, in a mouse model of thrombosis, a reduction of time needed for venous and arterial occlusion by a mechanism dependent on P-selectin. The same work demonstrates, by in vivo microscopy, that specifically derived tumor MPs bind to the thrombus through PSGL-1. Although a significant number of data and published clinical studies are in favor of a pathophysiological involvement of MPs in these tumor thrombosis-related cancers, a causal link has not yet been fully established in clinical practice.

### 3.2.4 Fibrinolytic activity of TMPs

The proof of concept of the role of MPs in the proteolysis was initially demonstrated from MPs derived from malignant tumor cells, which have an important proteolytic potential, characterized by the production of TMPs and uPA, necessary for their release and tissue invasion.

In a series of studies published in 1994, the presence of these proteolytic enzymes has been identified in vitro and in vivo, on the membrane of TMPs derived from surnagean culture of human tumor cell lines or ascites fluid obtain in patients with ovarian cancer.

The detection of the enzymatic activity by zymography and the carrier protein by immune footprint allows the identification on these MPs of urokinase (uPA) and urokinase receptor (uPAR), pro-matrix metalloproteinase-2 (proMMP-2), pro-matrix metalloproteinase-9 (proMMP-9) and their active forms, as well as complexes between Matrix metalloproteinases (MMs) and tissue inhibitor of metalloproteinase 1 (TIMP-1) inhibitor.

Proteolytic band having the molecular weight of t-PA was also detected by zymography in MPs from a carcinoma line of the prostate.

Extracellular matrix three-dimensional model (Matrigel) helped highlight a concerted action between uPA and pro-MMP of MPs in the degradation of matrix proteins in the presence of plasminogen.

The proposed mechanism suggested that the membrane of MPs operate by offering a wide TMPs activation surface via plasmin formation by uPA. These results were confirmed more recently by the study of isolated MPs collected ascites in patients with ovarian cancer.
This system of plasmin generation is controlled by the presence of inhibitor. An activity of PAI-1 has been described on the surface of cancer MPs \(^5\).

The urokinase is able to activate plasminogen on the surface of MPLs and derived cancer MPs. Plasminogen binds to the surface of the MPs by its two domains Kringles (K1 and K4) which have two sites of liason Lysine C-terminus (COO-) present on α-enolase. The uPA binds to its receptor uPAR and cleaves plasminogen at the same site (platelets, cells, fibrin matrix). Plasmin which remains partly bound to a surface of the MPs is protected from plasma inhibitors. This process is regulated by the α2antiplasmin (Fig. 28).

**Figure 28: Plasminogen activation on the TMPs surface**
*(From Lacroix et al. SeminThromb Hemost. 20)*
3.3 TMPs AS BIOMARKERS

In addition to the functions described above, there is emerging evidence for the role of extracellular vesicles in disease monitoring and diagnosis by serving as biomarkers in cancer. TMPs have been shown to have an incredible capacity to incorporate constituents from the parent cell and deliver them to recipient cells, contributing to cancer progression and resistance. It is this very capacity that makes TMPs a promising biomarker for the diagnosis, prognosis and surveillance of cancer.

Due to their presence in easily extracted body fluids and their capacity to reflect the characteristics of the parent cell, TMPs also have potential to be employed in diagnosis and prognosis. This may potentially circumvent the need in the future for invasive biopsy procedures in staging and grading of cancers. The determination of the molecular status of tumors allows for detection of specific disease markers, surveillance of cancer progression and supports approaches used in individualized and targeted therapies. This has potential for fast and efficient implementation of tailored interventions, resulting in improved clinical outcomes for the patient.

Clearly, more studies are needed to unravel the characteristics of such tumor stem-cell derived microvesicles, which may hold one of the keys that will unlock innovative approaches for future cancer treatment (Table 10).
Table 10: Tumor biomarkers in TMPs from body fluids of patients with cancer
(From Tumor-cell-derived microvesicles Martins et al.)

<table>
<thead>
<tr>
<th>Tumor</th>
<th>TCMV marker</th>
<th>Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>HER-2 oncprotein</td>
<td>Protein</td>
<td>Plasma</td>
</tr>
<tr>
<td>Stomach</td>
<td>HER-2/new, MAGE1, c-MET, and EMMPRIN</td>
<td>Protein</td>
<td>Plasma</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>EGFRVIII</td>
<td>Protein</td>
<td>Serum</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Caveolin-1, CD63, TYRP2, VLA-4, HSP70 and HS90 isoform, MET</td>
<td>mRNA/mRNA</td>
<td>Plasma</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
<td>Epstein-Barr oncoproteins (MP1 and BARF1)</td>
<td>Protein</td>
<td>Saliva</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>Prostate cancer antigen 2 (PCA2), TMFSS2, ERG [chromosomal rearrangement]</td>
<td>mRNA</td>
<td>Urine</td>
</tr>
<tr>
<td>Prostate</td>
<td>Deltocytin, miR-141 and miR-375 [advanced cancer]</td>
<td>Protein</td>
<td>Urine</td>
</tr>
<tr>
<td>Ovary</td>
<td>CD24, EpCAM, MMP2, MMP9, and ePA</td>
<td>Protein</td>
<td>Ascite</td>
</tr>
<tr>
<td>Claudin 4</td>
<td>MiR-21, miR-141, miR-200c, miR-200b, miR-203, miR-205 and miR-214</td>
<td>miRNA</td>
<td>Serum</td>
</tr>
<tr>
<td>Bone metastasis of prostate</td>
<td>Annexins A1, A2 and A5, dimethylarginine, dimethylaminohydrolase 1</td>
<td>Protein</td>
<td>Tissue</td>
</tr>
<tr>
<td>Colorectal</td>
<td>Overall levels of exosomes</td>
<td>Vesicles</td>
<td>Plasma</td>
</tr>
<tr>
<td>Pancreas</td>
<td>EGFR isoforms</td>
<td>Protein</td>
<td>Plasma</td>
</tr>
</tbody>
</table>

3.3.1 Diagnosis

Biomarkers for cancer screening and diagnosis often display low sensitivity and/or specificity, missing patients with early stage disease (false negatives) or detecting those with no disease (false positives).

TMPs may offer several potential benefits over current clinical biomarkers. In fact, MPs in cancer patients can exceed five-fold that in healthy persons\(^665\) \(^666\) \(^667\).

In healthy volunteers, the concentration of MPs is usually under 0.5 g protein/1 ml blood, while in cancer patients it reaches 2–5 g protein/1 ml.

Moreover, high rates of MPs were documented in solid cancers of various origins (colon, pancreas, breast, ...) but also in leukemia, including acute myeloid leukemia\(^668\).

TMPs may shuttle both clinically validated biomarkers [e.g., prostate-specific antigen (PSA)] and they are a novel source of proteins and nucleic acids that could be exploited as surrogate biomarkers\(^669\).

They protect their cargo from the attack of nucleases and proteases, increasing biomarker half-life, and potentially facilitating sample integrity and downstream molecular analyses.
particles are well suited for multiplexed biomarker analyses that may increase sensitivity and/or specificity of the diagnostic assay.

Clinical studies for TMPs-associated cancer biomarker shave been already described and they are summarized in (Table 11).

Mutations induce the formation of abnormal cells exhibiting uncontrolled cell division. Epigenetic modifications of gap junction proteins can lead to “communication problems” among cells, predisposing them to tumor formation.

There is clear evidence that TMPs release in plasma, urine, and other bodily fluids is enhanced in many cancers.

On the basis of the concept that TMPs contain ‘molecular signatures’ of their cell of origin, the systematic mapping of proteins, lipids, and nucleic acids in TMPs may provide a shortcut to identify specific biomarkers of cancer occurrence and metastasis.

To date, however, there has not yet been any significant prospective study on the value of quantitative or qualitative exosome analysis as a biomarker of early carcinogenesis.

There fore circulating TMPs have been detected in the blood, urine and ascites of cancer patients.

Logozzi and colleagues performed a retrospective study on TMPs-associated biomarkers in stage III and IV melanoma patients and they showed increased levels of caveolin-1- and CD63-positive TMPs.

TMPs-associated caveolin-1 displayed a sensitivity of 69% and specificity of 96.3% while levels of serum LDH were altered only in 12.5% of patients.

Mechanistically, TMPs may have a prominent role in the pathogenesis of melanoma. Melanoma cells have been shown to release exosome-associated oncoprotein MET to educate bone marrow progenitor cells and promote metastases in vitro and in vivo, and elevated levels of MET and phospho-MET have been detected in melanoma patients. Additionally, the authors showed aberrant levels of TMPs-associated biomarkers TYRP-2, VLA-4, HSP70, and HSP90 in the plasma of melanoma patients.

Indeed, HSPs are emerging as another potential source of EV-based cancer biomarkers. HSP70 is actively secreted by different types of tumor cells through non-classical protein secretory routes, including EVs, and HSP70-positive TMPs have been shown to activate macrophages and natural killer cells that act against cancer cells; while, the chaperone HSP90 has been shown to enhance cancer cell migration when is released by TMPs-derived cancer cells.
TMPs may be exploited as biomarker shuttles for the early diagnosis of prostate cancer (PCa). Serum PSA and prostate-specific membrane antigen (PSMA) have been found on plasma and urine-derived exosomes, though not validated in a large clinical study\textsuperscript{693}. In another report, exosomal survivin was identified as promising surrogate biomarker for early diagnosis of PCa\textsuperscript{694}. Plasma levels of survivin-positive-TMPs were higher in PCa patients than benign hyperplastic patients and healthy donors, potentially providing an alternative tool to reduce the number of false positives generated by the PSA test\textsuperscript{694} (Table 11).

<table>
<thead>
<tr>
<th>Cancer biomarker</th>
<th>Disease</th>
<th>Indication</th>
<th>Biofluid</th>
<th>Clinical study size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>Prostate cancer</td>
<td>Screening/early diagnosis</td>
<td>Urine</td>
<td>Controls $N = 10$; disease $N = 24$</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate cancer</td>
<td>Screening/early diagnosis</td>
<td>Plasma</td>
<td>Control $N = 2$; disease $N = 5$</td>
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<td>EGFRvIII</td>
<td>Glioblastoma</td>
<td>Early diagnosis</td>
<td>Serum</td>
<td>disease $N = 30$</td>
</tr>
<tr>
<td>PIMP</td>
<td>Melanoma</td>
<td>Early diagnosis/prognosis</td>
<td>Plasma</td>
<td>Controls $N = 7$; stage III $N = 24$; stage IV $N = 14$</td>
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<tr>
<td>Caveolin-1</td>
<td>Melanoma</td>
<td>Early diagnosis</td>
<td>Plasma</td>
<td>Controls $N = 58$; disease $N = 90$</td>
</tr>
<tr>
<td>Survivin</td>
<td>Prostate cancer</td>
<td>Early diagnosis</td>
<td>Plasma</td>
<td>HD $N = 8$; BPH $N = 20$; disease $N = 30$</td>
</tr>
<tr>
<td>CD 24</td>
<td>Breast cancer</td>
<td>Early diagnosis</td>
<td>Serum</td>
<td>HD $N = 14$, disease $N = 18$</td>
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<tr>
<td>EGFR</td>
<td>Lung cancer</td>
<td>Diagnosis/personalized medicine</td>
<td>Serum</td>
<td>HD $N = 9$, disease $N = 9$</td>
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<td>Ovarian cancer</td>
<td>Early diagnosis/prognosis</td>
<td>Serum</td>
<td>HD $N = 10$; stage I $N = 10$; stage II $N = 10$; stage III $N = 20$; stage IV $N = 10$</td>
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<tr>
<td>RNU6-1, miR-320, and miR-574-3p</td>
<td>Glioblastoma</td>
<td>Early diagnosis</td>
<td>Serum</td>
<td>Controls $N = 50$; disease $N = 50$</td>
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<tr>
<td>TMPRSS2:ERG2 and PCA3 mRNAs</td>
<td>Prostate cancer</td>
<td>Early diagnosis</td>
<td>Urine</td>
<td>Blinded prospective study $N = 30$</td>
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<tr>
<td>let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a</td>
<td>Colorectal cancer</td>
<td>Early diagnosis</td>
<td>Serum</td>
<td>Controls $N = 22$; disease $N = 88$</td>
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<td>miR-151a-3p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, and miR-154-3p</td>
<td>Lung cancer</td>
<td>Early diagnosis</td>
<td>Plasma</td>
<td>HD $N = 10$; benign disease $N = 10$; malignant disease $N = 10$</td>
</tr>
<tr>
<td>TGFBI and MAGI3/6</td>
<td>Ovarian cancer</td>
<td>Prognosis/therapy monitoring</td>
<td>Plasma</td>
<td>HD $N = 10$; benign disease $N = 10$; malignant disease $N = 22$</td>
</tr>
<tr>
<td>TYRP2, HSP70, HSC70, VLA-4</td>
<td>Melanoma</td>
<td>Prognosis</td>
<td>Plasma</td>
<td>HD $N = 9$; stage I $N = 2$; stage III $N = 7$; stage IV $N = 18$</td>
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<td>miR-21</td>
<td>Human esophageal cell carcinoma</td>
<td>Prognosis</td>
<td>Serum</td>
<td>HD $N = 41$; disease $N = 51$</td>
</tr>
<tr>
<td>KRAS</td>
<td>Pancreatic cancer</td>
<td>Personalized medicine</td>
<td>Serum</td>
<td>HD $N = 2$; disease $N = 2$</td>
</tr>
<tr>
<td>BRAFV600E, EGFR</td>
<td>Lung cancer, melanoma</td>
<td>Personalized medicine</td>
<td>Plasma</td>
<td>In vivo model $N = 8$</td>
</tr>
</tbody>
</table>

Table 11: Preclinical and clinical studies on Ev-Shuttled biomarkers
Analysis of the TMPs cargo may allow the repositioning of a clinically validated biomarker to a new diagnostic indication. For example, the FDA has recently approved a test (commercialized by Hologic) that supports the clinical decision of repeating a biopsy in suspected PCa patients.

The test is based on QRT-PCR detection of PCA3 mRNA from urine collected after digital rectal examination (DRE) and it is not recommended for the early diagnosis of PCa. Instead, a report from Dijikstra and colleagues suggested that the ratio between the levels of TMPs-associated PCA3 and PSA mRNAs might be useful for the early detection of PCa.695

TMPs have also been evaluated as diagnostic platforms for multiplexed approaches.

Seminal work by Taylor and colleagues identified the first disease-specific miRNA signature in derived from ovarian cancer patients.682

They identified a tumor-specific signature of eight miRNAs in EpCam-positive-EVs that discriminated ovarian cancer from benign ovarian disease.682 Remarkably, miRNA levels were not altered by preanalytical variables such as collection and storage time.682

In another retrospective study, a diagnostic signature of miRNAs was found and validated in serum derived EVs from colorectal cancer patients.696 Sensitivity of the signature was higher than 90%, while serum biomarkers CEA and CA19-9 displayed sensitivities of 30.7 and 16%, respectively.696

These studies provide some level of comfort to support further research around the clinical use of TMPs as biomarkers for screening and early diagnosis of cancer. However, they suffer from the lack of standardized protocols of sample collection and storage and the limited sample size.

For the former, some consensus was recently achieved by the International Society for Extracellular Vesicles, though not fully implemented in clinical studies yet.697

For the latter, large regulated multicenter clinical studies are needed to validate the use of TMPs for potential diagnostic applications.

Furthermore, elevated levels of circulating MPs have been detected in patients with non-small cell lung carcinoma and newly diagnosed glioblastoma patients compared to healthy controls.698 699

Additionally, breast cancer patients have higher levels of MPs than healthy controls or patients with benign breast tumors.700 701 702

This supports their potential as relevant diagnostic markers of malignancy.

In addition, the recent demonstration that next generation sequencing technologies can be applied to the genomic DNA and to miRNA enclosed in TMPs suggests the opportunity to derive tumor-specific genomic alterations from TMPs.643 703 704
Cancer patients not only exhibit tumor derived TMP but also high levels of platelet derived MPs.\textsuperscript{628 666 705}

The new approaches of liquid biology could represent a noninvasive method to detect TMPs and obtain diagnosis.

\textbf{Figure 29: Schematic representation of liquid biopsy oncology, in a sample of whole blood (A) or plasma (B).}

The samples were collected noninvasively and contain many information derived from diseased cells or tissues (cells, MPs, acids nucleic), whose detection will make a diagnosis, monitoring and propose individual treatment.

(From Graham and al. transl cancer res. 2015)

\textbf{3.3.2 Prognosis}

The detection of TMPs has been associated with the prognosis and clinical status of cancer patients.

TMP-based diagnostics may be a practical alternative to tumor biopsy diagnostics that are currently used for prognostic and “personalized medicine” indications.
Indeed, TMPs can be collected with minimally invasive procedures from a variety of body fluids; they may be more representative of the intra-tumor heterogeneity than fine needle biopsy, thus potentially revealing aggressive primary tumor features and distant metastases\textsuperscript{706}. These vesicles may allow the real-time monitoring of therapeutic responses and development of resistance mechanisms to targeted therapies where the mutation status is needed for patient stratification; EVs cargo may complement the use of other emerging “liquid biopsy” platforms such as circulating tumor DNA or circulating tumor cells\textsuperscript{703}. These studies lack the sample size and standard operating procedures required for clinical validation. However, the established presence of the disease may facilitate discovery and validation of cancer-derived biomarkers in these indications. Though the FDA has not approved TMPs-based tests yet, both industrial and regulatory entities have expressed their interest in developing TMPs-based tests for personalized cancer medicine. There are several compelling reasons for it. TMPs may represent a minimally invasive platform for the development of companion diagnostic (cDx) tests for targeted therapies. Cancer-derived TMPs may be exploited for the development of blood-based cDx for cetuximab (Erbitux) since they carry both the drug target (epidermal growth factor receptor) and the mutated KRAS gene, which correlates with poor therapeutic responses\textsuperscript{703}. Cancer-derived TMPs may also shuttle genomic DNA with the mutation BRAFV600E, which may be used to develop a cDx test to identify melanoma patients eligible for the treatment with Vemurafenib\textsuperscript{704}. Moreover, personalized medicine has become one of the fastest growing segments in the molecular diagnostic market due to FDA’s recent recommendation of developing cDx tests for approval of new drugs. From the perspective of diagnostic developers, cDx tests are very attractive since they may benefit from fast-track approval and positive clinical adoption. Finally, drug developers may decide to directly reimburse the cost of cDx test to drive clinical adoption and sales of the targeted therapy, thus relieving insurances and patients from a significant economic burden. Recently Wang et al showed in a study in end-stage patients with non-small cell lung cancer that the rate of MPs derived endothelial cells activated is predictive of mortality at one year\textsuperscript{707}. Another example is provided in pancreatic cancer.
Consistent with this was the strong association with thrombosis and increased mortality in patients with pancreaticobiliary cancers\textsuperscript{708}. This study amongst many others lends support to the diagnostic and prognostic potential TMPs have in cancer management. The prognostic role of TMPs was analyzed in other solid tumors, like prostate, hepatocellular cancer, breast cancer and glioblastoma\textsuperscript{709 710 711 712}.

3.3.3 Monitoring

With the rapid progress of targeted sequencing, it might also be possible, by analyzing the DNA content of circulating TMPs, to periodically monitor tumor progression and response to therapy, as well as investigate tumor heterogeneity, tumor evolution and clonality. More challenging is the study of the whole transcriptome in TMPs. In fact, current studies rely on the identification of specific mRNAs by polymerase chain reaction (PCR), whereas whole transcriptome profiling of TMPs has not been optimized\textsuperscript{594}. Reports on RNA sequencing often describe the relative proportion of different RNA species that can populate TMPs rather than identifying specific mRNA reads\textsuperscript{713 714}. Furthermore, because the predominant species seem to be ribosomal RNA and miRNA and other non-coding RN, the levels of mRNA might be extremely low\textsuperscript{715}. Therefore, for clinical applications of RNA profiling, it is important to develop protocols specific for TMPs and to achieve the deep coverage than that is commonly used to sequence tumor tissues.

Large oncosomes might represent a population of TMPs with advantages for translational studies. The detection of oncosome-like feature in tumor sections of patients with advanced disease can allow direct comparisons between tissue features and circulating large oncosomes in matched samples\textsuperscript{531}. As previously mentioned, large oncosomes might also contain more abundant tumor-derived molecules, possibly including actionable molecules, resulting in candidates for a non-invasive source of information useful in precision medicine. Importantly, analyses performed in circulating large oncosomes could be validated directly on tumor tissues.
3.4 TUMORIGENESIS AND TMPs

E. Pap et al. / Critical Reviews in Oncology/Hematology 79 (2011) 213–223

Figure 30: The role of TMVs in tumorigenesis.

MVs, shedding from the tumor cells promote tumorigenesis, transporting and transferring all those factors – proteins and non-proteins – which are in some way responsible for the survival and for the expansion of the tumor. This figure summarizes the potentials that lie in TMVs.
(From E. Pap et al. / Critical Reviews in Oncology/Hematology 79 (2011) 213–223)

2.4.1 Tumor growth and progression

Several reports suggest that they appear to be ubiquitously released by tumor cells, and the amounts shed increase with cell invasiveness or disease progression716.

The first key evidence for their role in disease progression came from the demonstration that vesicles shed spontaneously from highly metastatic B16 mouse melanoma cells (F10)—upon fusion with poorly metastatic B16 mouse melanoma cells (F1)—enabled F1 cells to metastasize
to the lung. These studies set the stage for further investigations into the importance of microvesicles in tumor progression. The survival of malignant cells is sustained by different escape mechanisms: the absence of adequate immune responses or the induction of immune tolerance as well as the potential to escape apoptosis. The expansion of the tumor is associated with remodelling of the extracellular matrix and with angiogenesis.

Tumor derived TMPs can functionally modify fibroblasts, by reprogramming these cells to cancer associated fibroblasts (CAFs), which exhibit a myofibroblastic differentiation.

Studies from Webber et al. demonstrated that exosomes can release a TMP specific form of transforming growth factor beta1 (TGF-β1), which differs from the soluble form of this growth factor in that not only does it induce myofibroblast differentiation but also actively promotes tumor progression.

Interestingly, disruption of TMPs mediated interactions of the tumor cells with the surrounding stroma in vivo significantly reduces tumor growth by impairment of either TMP-dependent signaling activation in target cells or exosome production.

This result might imply that TMPs could be targeted at multiple levels, from biogenesis to interactions with target cells, alone or as combinatorial approaches. Parallel studies, from Antonyak et al., have demonstrated that MVs released from breast cancer or glioblastoma cell lines can induce transformation of fibroblasts and the process is mediated by fibronectin1 (FN1) and transglutaminase (tTG).

Along with the notion that CAFs might release soluble factors that induce epithelial-mesenchymal transition or stemness in cancer cells, it is becoming evident that these processes might be also regulated by CAF-derived TMPs.

TMPs can facilitate the progression of the tumor by both autocrine and paracrine signalling. They transfer growth factors receptors, mRNA and miRNA, thereby they can modify the phenotype and the nature of the cells, even in the lack of earlier mutagenic events.

Several groups described the so-called horizontal transfer by TMPs between cells. Skog and his group showed that glioblastoma TMPs, isolated from the serum of patients suffering from brain tumor, transport RNA and proteins that promote tumor growth.

Several oncogenic activities have been attributed to the truncated form of epidermal growth factor receptor EGFRvIII found in glioblastomas. Among them are activation of MAPK and Akt pathways and reprogramming of EGFRvIII regulated gene expression (VEGF, Bcl-x (L), p27).

Working in mice, Al-Nedawi and co-workers showed, that although EGFRvIII is expressed
only in a small percentage of the glioblastoma cells, it will be transferred through TMVs to other brain tumor cell subsets. Therefore, this horizontal transfer leads to the spreading of oncogenic activity. They obtained similar results later with human cancer cell-derived TMVs, which displayed EGFR.

These TMPs were taken up by endothelial cells and increased the expression of VEGF, which elicited furthermore an autocrine activation on the expression of VEGF receptor-2. So far, however, there is still only fragmented information on how tumor-associated metabolic changes may influence the production and biological activity of TMPs on tumor progression and growth.

In recent years, cancer has emerged as a disease of metabolism and cell bioenergetics, highlighted by the capacity to ferment glucose into lactate regardless of the presence of oxygen, an effect known as ‘aerobic glycolysis’ (Warburg effect). Oxygen deprivation is a limiting factor for solid tumor growth, and mild-to-severe hypoxia is responsible for aberrant neoangiogenesis.

Thermal and oxidative stresses increase the exosomal secretion of ligands of NKG2D (NK cell receptor natural killer group 2, member D) by T-cell and B-cell leukemia/lymphoma which downregulates NKG2D receptor-mediated response, iTMPairs Nkcell cytotoxicity, and contribute to immune escape. Conversely, TMPs from heat-stressed tumor cells contain chemokines such as CCL2, CCL3, CCL4, CCL5, and CCL20 that chemoattract CD11c(+) DC and CD4(+)CD8(+) T-cells which activate a specific antitumor immune response in tumor-bearing mice.

Other stress conditions such as treatment of breast cancer and erythroleukemic cells with INFg induce the secretion of exosomes containing the heat shock protein HSP72, which upregulates CD83 expression and stimulate IL-12 release by naive dendritic cells, enhancing tumor recognition.

An important metabolic process influencing microvesicles biogenesis and tumor progression is autophagy, the main physiological pathway for lysosomal degradation of malfunctioning macromolecules and obsolete or damaged organelles in eukaryotic cells. Stress-mediated autophagy triggers either protumorigenic or antitumorigenic effects, depending on cellular and environmental context. Under nutrient deprivation, autophagy recycles proteins into bioenergetic metabolism and contributes to cell survival. In other contexts, autophagy promotes stress tolerance facilitating cell survival and tumor growth. One of the intracellular vesicle structures of the autophagic pathway, the autophagosome, can fuse to MVB and other endocytic structures to generate the amphysome, which fuses to
lysosomes. Thus, extracellular signals that increase the interactions between autophagosomes and MVBs can block exosomal secretion\(^7\)\(^{26}\). Whether regulation of exosomes secretion by autophagy prevents tumor and metastasis signaling by TMPs remains to be demonstrated.

The p53 protein, the product of the tumor suppressor gene TP53, interconnects stress responses, metabolism, and exosomal secretion. The p53 protein is activated in response to many forms of stress that induce DNA damage and control the transcription of a series of genes involved in cell cycle, apoptosis, senescence, differentiation, and basal energy metabolism, thus contributing to multifaceted antiproliferative effects\(^7\)\(^{27}\). Tumor suppressor activated pathway 6 (TSAP6) is a transcriptional target of p53, which encodes a multipass membrane protein involved in exosome secretion. Mice deficient for TSAP6 exhibit severely compromised exosome production\(^7\)\(^{28}\). There is a paradox, however, in the fact that the levels of exosomes in the plasma are apparently higher in patients with colorectal cancer containing TP53 mutations than in matched patients with wild-type TP53, even though the former has lost transactivation of TSAP6 by p53.
Figure 31: The pleiotropic effects of TMVs on the growth and survival of the tumor.

TMVs provide a pleiotropic effect: they influence other tumor and non-tumor cells and remodel the extracellular matrix, in order to escape immune cells and drugs, also to “serve” the establishment of a favorable environment for tumor.

(From E. Pap et al. / Critical Reviews in Oncology/Hematology 79 (2011) 213–223)

3.4.2 Metastasis

Metastasis is an especially unfavorable aspect of cancer progression, whereby a localized population of cancer cells spreads through the lymphatic system or bloodstream to other parts of the body.

The “success” of tumor cells to form metastasis greatly depends on their ability to anchor to the extracellular matrix, to degrade it and to migrate and be attached to a new surface. The process necessitates formation of new blood vessels. Adhesion proteins and their receptors, extracellular matrix proteases, inducers of vascularization have been observed in TMVs generated by many sorts of cancer. For example, a recent study has reported that normal human endothelial cells can be stimulated to form vessels in vivo by TMPs derived from a subset of tumor-initiating cells expressing the mesenchymal stem-cell marker CD105 in human renal cell carcinoma. These TMPs enhanced lung metastasis and contained a specific set of proangiogenic mRNAs and microRNAs implicated in tumor progression and metastasis. Moreover, Lima and co-workers reported that TMPs of the B16F10 melanoma cell line induce melanoma metastasis, which are normally resistant to this cell line. In addition, the group of Janowska-Wieczorek found horizontal transfer among platelet-derived MVs and human lung and breast cancer cells, transferring platelet-derived adhesion molecules to them, conferring a strong metastatic potential upon them.

Degradation of extracellular matrix is an essential process in tumor expansion. In fact, to facilitate invasion, metastatic cancer cells can degrade the extracellular matrix (ECM) through the transfer of surface proteases such as matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and cathepsins. Tumour cells have been shown to release TMPs rich in MMPs and uPA, which degrade the ECM, allowing for cell invasion. Among the many biologically active constituents of TMPs are extracellular matrix metalloproteinase inducer, MMPs, tissue factor (TF), VEGF, HGF, CD44 and 1-Integrin. These components of TMPs stratify the malignant abilities of other tumor cells through the horizontal...
transfer of effector molecules. In addition, they can stimulate matrix metalloproteinase expression in fibroblasts and consequently facilitate tumor invasion and metastasis. Moreover, these TMPs-components reach distant areas and prepare the favorable microenvironment for the migrating malignant cells. For example, ovarian cancer cells secrete TMPs and promote an angiogenic phenotype in endothelial cells in vitro through a mechanism mediated by CD147 (matrix metalloproteinase MTMP inducer or basigin). MMP-2 and MMP-9 were found in MPs released from ovarian cancer cells but also from breast cancer cells and enhanced the metastatic capacity of recipient cells. Moreover, TMPs from prostate and lung carcinoma cells activate stromal fibroblasts and endothelial cells to upregulate MTMP increasing the motility, resistance to apoptosis, and production of angiogenic factors. In addition, leukemic B-cells deliver TMPs that interact with stromal cells promoting cyclin D1, c-myc, and VEGF expression.

The secretion of TMPs is largely increased during hypoxia and contributes to tumor progression. Under oxygen deprivation, ovarian and endometrial carcinomas secrete large amounts of TMPs containing the adhesion molecule L1, a potent inducer of cell migration. Increased levels of TMPs also occur in hypoxic lung carcinoma cells. These vesicles activate and chemoattract stromal fibroblasts, and endothelial cells induce the expression of angiopoietic factors in stromal cells and stimulate the latter cells to secrete factors that enhance the metastatic potential of tumor cells in vivo.

MPs are a conduit between drug resistance and metastasis. This appears to be mediated via the regulation of miR-503 and proline-rich tyrosine kinase 2 (PYK2) to promote the migration and invasive capacity of recipient breast cancer cells. Specifically, the authors confirmed that miR-503 is required for the inhibition of migration and invasion in breast cancer as demonstrated by wound healing migration assays and invasion assays. This finding supported previous observations of reduced migration and invasion following transfection of miR-503 in hepatocellular carcinoma cells, acute myeloid leukemia cells, chronic myelogenous leukemia cells, osteosarcoma cells, colon cancer cells, head and neck carcinoma cells and in endometrioid endometrial cancer cells. Moreover, the authors showed for the first time that MPs were involved in mediating the effects of miR-503 in breast cancer cells. One such mechanism for the down regulation of miR-503 by MPs was proposed to be via the activation of the NF-κB signaling pathway, as NF-κB has been shown to suppress the expression of miR-503 in epithelial cells.

Furthermore, PYK2 protein and mRNA was found to be upregulated in recipient cells following co-culture with MPs. PYK2, a member of the focal adhesion kinase subfamily of
cytoplasmic tyrosine kinases, was correlated with an increased metastatic capacity in a breast cancer cell line, a squamous cell carcinoma of the head and neck, hepatocellular carcinoma and prostate cancer. Both PYK2 and miR-503 may promote metastasis in recipient cells via regulation of the PI3K/AKT signaling pathway. The overexpression of PYK2 was associated with activation of the PI3K/AKT pathway and poor survival and metastasis in hepatocellular carcinoma. Additionally, as miR-503 targets and inhibits PI3K/AKT activation, the suppression of miR-503 in recipient cells may result in the up-regulation of PI3K/AKT signaling and the subsequent promotion of metastasis. Although up-regulated in recipient cells, PYK2 was not found in the cargo of the MPs themselves. This was the first demonstration that it was the dissemination of intermediary mediators that led to the MP-mediated regulation of PYK2 in recipient cells rather than the direct transfer of components packaged within the MP cargo. Therefore, the scope of MP involvement in the promotion of migration and invasion is continuously broadening.

Microvesicles play critical roles in either enhancing or decreasing metastasis by operating as signaling platforms that shuttle molecular information between tumor cells and their environment. Tetraspanins, a family of transmembrane proteins commonly detected in MPs, can recruit a variety of proteins and modulate their cognate signaling pathways. For example, the tetraspanins CD82 and CD9 sequester E-cadherin/beta-catenin complexes within exosomes, decreasing their cellular availability and thus downregulate epithelial-to-mesenchymal transition (EMT) leading to metastasis. In contrast, MPs may also operate as presenting particles for metastasis-promoting factors such as amphiregulin, which is at least five times more potent in activating EGFR when associated to microvesicles than in its soluble form. The concept of premetastatic niche is based on the notion that metastatic cells require a receptive microenvironment supporting a temporal sequence of events that favors their arrival, engraftment, and survival in the metastatic site. In recent years, evidence has accumulated that MPs have a key role in the initiation of the premetastatic niche through their capacity to promote communication between tumor and nontumor cells. MPs contain molecules recognized by T-cells, which may exert immunosuppressive effects facilitating immune escape of metastatic cells. The demonstrated TMP ability to enter the circulation and potentially travel far from the site of their origin has generated the hypothesis of a role for MPs in the education of the metastatic niche. In vivo results in this area are still largely limited. However, Peinado et al. have reported, in a very elegant study, the capacity of melanoma-derived MPs within the bone marrow to condition the metastatic niche. Moreover, CD105 positive MVs, which can promote angiogenesis in vitro, may stimulate lung metastasis in vivo.
The role of TMPs in promoting metastasis has recently received spectacular in vivo support when it was demonstrated that exosomes derived from melanoma cells can home to sentinel lymph nodes, modifying the microenvironment and promoting the expression of sets of genes that facilitate the recruitment, trapping, and growth of tumor cells. These TMPs also increase vascular leakiness in premetastatic niches influencing the intravasation of tumor cells and their metastatic growth.

In combination with several cytokines and soluble factors, TMPs act as systemic messengers influencing the route of dissemination of metastatic cells and contributing to tumor-specific patterns of secondary cancer lesions. The microvesicle-mediated signaling processes that promote metastasis are not only supported by TMPs, but also involve signaling through microvesicles secreted by stromal, immune response, inflammatory, and vascular cells in the microenvironment. For example, in a mouse model carrying xenografts of cancer cell lines, exosomes produced by the activated T-cells promote the invasive ability of B16 murine melanoma cells and their migration to the lung. Thus, microvesicle-mediated signaling that promotes metastasis involves multidirectional trafficking of microvesicles between normal, activated, and tumor cells, defining a complex interactome of microvesicles that primes the metastatic niche, enhances migration, and facilitates immune escape.

Figure 32: Principal stages in the formation of a metastasis
3.4.3 Angiogenesis

Sphingomyelin carried by tumor MPs could be a pro-angiogenic active component, promoting cell migration, neovascularization and tumor invasion\textsuperscript{313 570}. In particular, MPs expressing the neutral sphingomyelinase 2 (nSMase 2) can induce tubule formation and migration of HUVEC and promote tumor progression through \textit{in vivo} stimulation of angiogenesis as result of their interaction with endothelial cells\textsuperscript{760}. Formation of a vasculature within the tumor facilitates cancer cell entry in the circulation thus promoting metastasis. The demonstration of a direct involvement of tumor derived-MPs in this process might provide additional targets for the development of anti-angiogenic drugs that may be used in combination with the ones already developed. In addition to the direct transfer of canonical pro-angiogenic proteins, recent studies are identifying novel TMP-enclosed molecules that participate in angiogenesis. These include EGFR, miR-210 and miR-9\textsuperscript{645 720 761}. Angiogenesis is strongly stimulated by hypoxia, in which an increased release of exosomes stimulates tubule formation in different tumor types\textsuperscript{645}. The presence of PPAR\(\alpha\) on the surface of circulating MPs also stimulates angiogenesis via signaling pathway Akt-NFkB\textsuperscript{762}. Similarly, the FT carried by MPs is a powerful regulator of angiogenesis, it increases the synthesis of VEGF (vascular endothelial growth factor)\textsuperscript{763}. It was also shown that MPs from endothelial progenitor cells stimulated angiogenesis by horizontal transfer microRNA\textsuperscript{764}. The MEPs stimulate tissue repair mechanisms has been shown in the muscle tissue, this effect is dependent on a rise beyond differentiation of endothelial progenitor cells, by a mechanism involving the generation of oxygen free radicals\textsuperscript{321}. Stromal fibroblasts and endothelial cells form part of an interacting network, in which information is largely transmitted by MVs.

Studies on the role of TMPs in angiogenesis have demonstrated that cancer cell-derived TMPs contain interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF), potent pro-angiogenic factors, as well as other molecules able to enhance endothelial cell invasion and organization in tubule-like structures\textsuperscript{594 639}. In fact, in human and murine lung cancer cell line derived TMPs chemoattract endothelial cells directly, which then secrete more IL-8 and express more ICAM (intercellular adhesion molecule) and VCAM (vascular cell adhesion molecule). These TMPs promote angiogenesis through stroma cells indirectly as well, inducing them to
express several pro-angiopoietic factors\textsuperscript{615}. Tissue factor is extremely highly expressed in tumor cells. TMPs bearing TF may trigger systemic coagulopathy and transfer TF activity among various cells\textsuperscript{765}.

3.4.4 Escape of TMPs from immuno surveillance

EVASION FROM IMMUNO SURVEILLANCE

In order to evade the protective surveillance of the organism, the escape mechanisms of the tumor cells include failure in tumor antigen presentation, expression of proapoptotic signals and inhibitory cytokine secretion, possibility to induce immune tolerance and insensitivity to anti-tumor drugs.

Tumor antigen presentation can be damaged by the increased rate of TMP shedding, resulting in elimination of most tumor antigens. Consequently, cytotoxic T lymphocytes and NK cells fail to recognize the oncogenic cells. In addition, TMPs can enhance immune tolerance by disseminating self-protective non-classical MHC complexes e.g. HLA-G and HLA-E. They are expressed and presented in tumor cells, travel in the membrane of TMPs and fusing with neighboring or distant tumor cells, they propagate the protection of less protected cells, thus they induce immune tolerance\textsuperscript{525, 766}.

The immunosuppressive effect of TMPs was partially revealed by the group of Valenti\textsuperscript{522, 528}. Her group performed studies on TMPs, deriving from cancer cell lines, patients with melanoma, colorectal cancer, head and neck tumor and ovarian carcinoma. When these TMPs were added to monocytes, they skew the process of normal differentiation into defective dendritic cells which then bearded low levels of CD80, CD86, HLA-DR co-stimulatory molecules, or even lacked them. In addition, the cells started to secrete TGF spontaneously. These changes inhibited T cell proliferation and functions.

The ability of TMVs to induce suppressive and “anomalous” immune responses was demonstrated by Clayton and, his group as well. He proposed the so-called “exosomes’ double hit to cellular immunity” theory. They treated IL-2 induced NK, CD8+ and CD4+/25+/foxp3+ T cells with exosomes collected either from mesothelioma tumor cell line from cancer patients or from Jurkat and leukemia cell lines.

The ability of NK and CD8+ cells to fight against tumor cells was demolished, they stopped proliferating and differentiating.

At the same time, the immunosuppressive effect of CD4+/25+/foxp3+ regulatory T cells was
enhanced as a consequence of the expression of the membrane-associated form of TGF1. It must be noted that membrane-bound TGF has approximately 1.4 times greater efficacy than the soluble form, perhaps a result of sustained signaling through the membrane-bound contact of the TMP and the recipient cell. Evidently, the powerful bioactivity of the membrane-inserted proteins contributes to this phenomenon.

In inflammation, macrophages help to create a beneficial microenvironment for tumor cells through their TGF1 secretion as well. TGF inhibits not only the proliferation/differentiation of T and B cells, but also the induction of tumor cell apoptosis. Exacerbating the situation, as described in previous paragraphs, tumor cells exhibit high levels of PS, and therefore attract and stimulate macrophages. TMPs display PS as well, so macrophages from distant loci are activated and recruited to the nascent tumor and help to create a microenvironment optimal to the survival of tumor cells. Studies on the highly metastatic B16F10 melanoma cell line suggest that melanoma cell-derived TMPs exert anti-inflammatory and immunosuppressive activities.

Direct interactions between TMPs and human monocytes/macrophages were described by Baj-Krzyworzeka. The authors could show that TMPs, isolated from the cell lines of pancreatic, colorectal adenocarcinoma and lung carcinoma, transferred CCR6 and CD44v7/8 to monocytes, which resulted in the alteration of their biological activity. These studies imply that TMPs affect not only existing and potential tumor cells, but different cell types in the “niche” of the developing tumor. By altering the biological activity of those cells, a tumor-favoring or/and an anti-tumor milieu will be set up. It is not clear though what kind of advantage can be obtained for the tumor cells by the release of TMPs provoking an anti-tumor effect in monocytes. It is to be noted that the previously described results were obtained from cancer cell lines in vitro, and supposedly, in vivo, the monocyte induced interact between the cells and the MPs from all kind of origin, present in each tumor environment. Presently it is quite difficult to roll up all in vivo interactions in the cellular–microvesicular network.

One further form of the escape machinery, established by tumor cells, is the induction of apoptosis of T cells by proapoptotic molecules expressed on tumor cells. It is reasonable to suppose that TMPs can also induce T cell apoptosis.

Indeed, TMPs express FasL and/or TRAIL, suggesting that the TMPs themselves contribute to the spread of immunotolerance towards tumor cells. Valenti’s group showed that when anti-tumor T cells were treated with TMPs, FasL and TRAIL sensitive tumor-specific T cells underwent apoptosis. Similar results were found with FasL+TMPs from patients with active
oral squamous cell carcinoma; treatment with these structures induced apoptotic pathways in Jurkat cells and in activated T cells from peripheral blood. The apoptotic effect of TMPs has been attributed to their property of downregulating the expression of CD3 and Janus-activated kinase-3 in T cells.

Tumor cells do not just induce cell death of other immune cells; they escape it themselves. One way to escape cell death was described in K562 erythroleukemia cells. The membrane attack complex (MAC) accumulates in the cell membrane, but through vesicle shedding the cells can avoid complement-induced cell death. Other strategies of the tumor cells are also supposed to be beneficial to them. Different mechanisms by which cells evade apoptosis have yet to be demonstrated in tumor cells per se. Platelets and endothelial cells, for example, form large quantities of MVs in response to internal stress. This way they can get rid off caspase-3 proteins, which prevents the cell from undergoing apoptosis. When MV formation was inhibited, the caspase-3 content remained high and the cells underwent apoptosis.

Tumor cells can acquire immunotolerance either through induction of T regulatory (T reg) proliferation or cytotoxic T cell death, and both mechanisms can be mediated by TMPs. In fact ovarian cancer-derived TMPs can enhance Treg proliferation and activity, and TMPs originating from tumor cell lines can induce a FasL or TNF-related apoptosis-inducing ligand (TRAIL) dependent cell death in CD8+ T cells. Similar results have been elicited by TMPs obtained ex vivo from patients with oral squamous cell carcinoma. However, exosomes may not always be involved in the regulation of the adaptive immune response in vivo. This finding highlights the difficulties in translating in vitro findings to animal and human models due to large intercellular diversity in vivo.

ELUSION OF APOPTOSIS

TMPs have been shown to contribute to tumour survival and it has been postulated that tumour cells can evade apoptosis by releasing TMPs that contain caspase-3, thereby preventing its intracellular accumulation. Inhibition of this release has been shown to result in caspase-3 accumulation and subsequent apoptosis.
Figure 33: Role of apoptosis in cancer

a | Tumour-initiating mutational events such as oncogene activation promote cell proliferation, but also apoptosis, which limits tumour growth. Following the acquisition of defects in apoptosis, tumour proliferation is sustained in the absence of apoptotic cell death.

b | Tumour growth is initially limited by the absence of a blood supply, which can trigger autophagy-mediated survival in the most metabolically stressed tumour regions, commonly the hypoxic center. The eventual recruitment of a blood supply cures the tumour of hypoxia and metabolic stress, and the tumour cells formerly surviving through autophagy can emerge to contribute to tumour growth.

c | In tumours formed by cells with defects in both apoptosis and autophagy, necrotic cell death is stimulated in metabolically stressed tumour regions and this necrosis is associated with the activation of an inflammatory response, DNA damage and tumour progression. Analogous to a wound-healing response, chronic necrosis and inflammation can stimulate angiogenesis and tumour growth.

(From Robin Mathew, Vassiliki Karantza-Wadsworth & Eileen White Nature Reviews Cancer 7, 961-967 (December 2007))

Researchers suggest, that an increased TMPs formation in tumor cells would lead to the loss of caspase-3, so cell death would be avoided.

In addition, caspase-3 may serve as an important factor for cytoskeletal reorganization, thus
may have a role in MV budding. The lack of caspase-3 was found to interfere with apoptotic body release.

MCF-7 breast cancer cell line does not contain functional caspase-3, due to a deletion in an exon. These cells do not display the typical morphological membrane changes prior to apoptosis. When transfected with a construct encoding caspase-3, they start to show membrane blebbing\textsuperscript{778}. Although the mechanism is not understood, it may be associated with the influence of caspases on the integrity of the cytoskeleton.

### 3.4.5 Drug resistance

Multidrug resistance (MDR) is the phenomenon by which cancers become cross-resistant to a wide variety of functionally and structurally unrelated chemotherapeutic drugs and constitutes the most significant obstacle in successful cancer treatment\textsuperscript{661,779}. MDR is predominately associated with the over-expression of the multidrug efflux transporters P-glycoprotein (P-gp/\textit{ABCB1}) and Multidrug Resistance-Associated Protein 1 (MRP1/\textit{ABCC1}), which are both expressed in the cell membrane of resistant cancer cells\textsuperscript{780}. These transporters belong to the superfamily of ATP-binding cassette (ABC) proteins which have also been implicated in infectious diseases such as AIDS and malaria\textsuperscript{781,782}. P-gp and MRP1 enable the maintenance of sublethal intracellular concentrations of chemotherapeutic drugs by facilitating the ATP-dependent efflux of anticancer agents, allowing the tumour to evade the toxic insult\textsuperscript{783,784,785}. The mechanism that allows these transporters to bind and efflux a broad repertoire of drugs remains yet undefined.

Since the discovery in 1981 that verapamil and trifluoperazine could positively affect MDR by the modulation of P-gp, hundreds of drugs with different structures and activities, such as immunosuppressants, calcium channel blockers and steroids have been developed\textsuperscript{786,787}. Inhibitors of P-gp have been investigated in the prevention or circumvention of MDR clinically, with many showing positive effects \textit{in vitro}, but proving to be unsuitable clinically due to dose limiting toxicity and lack of efficacy\textsuperscript{788,789}. A wide-spread strategy by which tumor cells resist drug therapy is to “ignore” it. The effectiveness of drug therapy is dramatically reduced in tumors from certain patients.
One cause is that this resistance to drugs is due to multidrug resistant (MDR) drug-efflux pumps, such as Pglycoproteins.

It has been shown that drug-sensitive (MDR−) cells also acquire the MDR phenotype subsequently, not by an increased expression rate, rather by an intercellular transfer of P-glycoprotein. This transfer is suggested to be via TMPs790,791, thus TMPs are capable to spread drug resistance to those tumor cells, which had not sustained higher expression earlier. Another option to ruin drug efficiency is to sending them out in TMPs. Certain drugs were found to accumulate in vesicles, which then shed off the cells. For example, ovarian carcinoma cells eliminate doxorubicin via exosomes792.

The involvement of TMPs in drug resistance is a novel area of investigation. TMPs from breast cancer cells and other tumor types can transfer resistance to docetaxel in cells that are sensitive to the drug641,793.

In breast cancer the process seems to be specifically mediated by P-glycoprotein, or by its activator transient receptor potential channel 5 (TrpC5)641,794.

TMP-mediated docetaxel resistance is also regulated by miR-222795. Further evidence demonstrates that the transfer of the pro-survival Akt/mTOR coTMPlex in TMPs results in propagating resistance to gefitinib in non-small cell lung carcinoma cells (NSCLC)793.
Figure 34: TMPs link the development of drug resistance to an enhanced metastatic capacity in cancer.

(A) TMPs shed from highly metastatic, drug-resistant donor cells transfer components such as P-gp protein, mRNA and associated miRNAs, PYK2 and miR-503 to up regulate P-gp expression and metastatic capacity in lowly metastatic, drug-sensitive recipient cells. (B) Recipient cells acquire both drug resistant and metastatic traits to promote the evasion of chemotherapy and metastatic spread of cancer.

(From J. Gong et al. / Seminars in Cell & Developmental Biology 40 (2015) 35–40)
3.5 MICROPARTICLES AND CANCER THERAPY

3.5.1 Against TMPs

Considering that MVs are involved in tumorigenesis at multiple levels, and that drugs themselves can be expelled from tumor cells via TMPs, interfering with the formation, release and propagation of these vesicles can be a novel and alternative issue in cancer treatment. Potential strategies in MV-related tumor therapy include: influencing MV formation and release, removal or inhibition of TMPs.

Inhibition and block of TMPs

The subject of TMP inhibitors is an emerging focus in the field. Calcium channel blockers, ROCK inhibitors and pantethine have been shown to prevent the production and release of TMPs in various cell types. This new class of compounds has potential to provide a novel strategy in circumventing TMP-mediated dissemination of deleterious traits and preventing cancer progression.

Using proton pump inhibitors (PPIs), TMPs formation can be blocked. In order to restrain ceramide accumulation needed for TMPs sorting, the inhibition of the ceramide synthesizing enzyme sphingomyelinase leads to blocking TMP release.

The spectrum of protein which may represent a therapeutic target to prevent TMP formation and release is extremely broad. However, as normal vesicle physiology is a prerequisite for life in all cells, the usability of such a drug would be uncertain, unless the most appropriate and tumor-specific agent would be found. Broad effects will have adverse side effects in healthy cells.

Promising strategy to inhibit TMP production are for example calpain inhibitors, calcium channel blockers, and ROCK inhibitors.

Removal of TMVs from the patient’s blood

Ichim and his group proposed a physical approach in order to remove tumor TMPs from the patient’s body fluids. The method is an extracorporeal “dialysis” through a "so-called" Hemopurifier TMPs, which is a hollow-fiber cartridge, that was originally designed to eliminate heavily glycosilated surface proteins and viruses. Tumor cell membranes and immunosuppressive TMVs are also highly glycosylated and thus, bind preferentially to the resin compared to nonmalignant cells. Furthermore, attaching specific molecules or antibodies
to the cartridge, the selective removal of targeted TMV scan be increased. This method combines dialysis and affinity chromatography. It has been used in clinical trials. The immunosuppressive activity normally found in the ascites fluid of patients with ovarian cancer were completely removed by the Hemopurifier TMPs. The authors propose that this method can be a novel, easily implemented approach in cancer therapy.

3.5.2 TMPs as antiblastic drugs

The capacity of TMPs to carry a multitude of components as part of their cargo can also be exploited in drug delivery. Since TMPs naturally function as vehicles for the delivery of bioactive molecules, the refinement and modification of these processes may allow TMPs to be used as novel therapeutic vehicles in the treatment of cancer.

A number of this P-gp is inside-out in orientation such that rather than its traditional function of effluxing drugs, this results in the actively influx of drugs into TMPs where they become trapped. Such a mechanism for drug trapping may be harnessed for therapeutic use in a similar manner to that employed by synthetic liposomes.

Indeed, a study conducted in 2012 showed that malignant cells incubated with chemotherapeutics drugs could package these drugs in the TMPs released from them. These drug-loaded TMPs in turn were shown to have an anti-tumor effect in murine tumor models with-out the typical side effects.

The development of TMPs as a viable mechanism for the delivery of therapeutic molecules would be advantageous over artificial vesicles as they can be isolated from the patient, loaded with the desired drug(s) and reintroduced into the patient during treatment. Therefore, complications associated with rejection and immuno-genicity would be avoided using these autologous and biocompatible vehicles. The potential for using such vehicles for therapeutic delivery has been described for exosomes.

The clinical viability of dendritic cell-derived exosomes was assessed in Phase I trials with melanoma patients and in patients with on-small cell lung carcinoma, with results showing that the therapy was well tolerated and could produce the required therapeutic effects. There is mounting interest in the delivery of nucleic acids for cancer therapy using this same approach. Loaded MPs were used to deliver exogenous short interfering RNA to the brain tissue of mice, resulting in specific gene knockdown of BACE1, a therapeutic target in Alzheimer’s dis-ease, and reduction in -amyloid 1–42 levels, a component of the amyloid plaques associated with
Alzheimer’s disease. Further-more, microvesicles harboring suicide gene mRNA and protein from donor cells reduced the growth of schwannoma tumors in an orthotopic mouse model. TMPs have also been shown to selectively package miRNAs and deliver them to recipient cells to regulate target gene expression and cellular function. Therefore, there is extensive potential to use endogenously or exogenously loaded MPs in gene therapy as part of cancer therapy.

The immunotherapeutic effect of ascites derived exosomes in combination with GM-CSF has been assessed in Phase 1 trials for the treatment of colorectal cancer. In this study, exosomes could induce an antigen-specific anticancer cytotoxic T lymphocyte response inpatients, with minimal toxicity and tolerated administration.

Likewise, mesenchymal stem cells (MSC) derived TMPs were shown to induce cell cycle arrest and apoptosis of different tumor cells as well as inhibit in vivo tumor growth. This approach is potentially beneficial as TMP inhibition of disease progression occurs in the absence of MSC differentiation into stromal fibroblasts which would otherwise be conducive to tumor growth.

Several studies have attempted to identify signatures of cancer and cancer progression and response to the therapy using proteome and miRNome profiling. For example, reduced levels of miR-34a in TMPs derived from docetaxel resistant prostate cancer cells and of miR-192 in TMPs derived from lung adenocarcinoma have been recently proposed as possible indicators of cancer progression and metastasis.

**MVs as vaccines**

Several teams examined the capacity of MVs to modulate the immune system. TMPs efficiently transfer tumor antigens from tumor cells to other antigen presenting cells. These antigen presenting cells, most importantly dendritic cells (DCs), promote initiation and amplification of anti-tumor immune response.

On the same principle as vaccination tumor antigens could be presented to the immune system to trigger an immune response against the tumor while not itself considered non-self. Theoretically TMPs would be equally suitable for this kind of treatment because they already present tumor antigens on their membrane. However, currently no immunotherapy treatment has been tested in clinical trials.

“Active vaccination” consists of injecting the patient with dendritic cell-derived TMPs (DEX) loaded with tumor peptides. These DEXs have been purified from DCs, cultured after leukapheresis of the melanoma patients, then they were loaded with tumor peptides and
reinjected into the patients.

An alternative method of “active vaccination” starts with the isolation of ascites-derived TMPs (ExAS) from a patient with ovarian carcinoma. They will be supplemented with adjuvants and reinjected into the patient.

In both cases the vaccines (the TMPs) are injected into the subcutaneous tissues at different sites, far from the already existing tumor’s immunosuppressive environment, with a strong antigen presentation potential. They provoke a strong and targeted anti-tumor T cell response. A tumor-derived exosome-mediated tumor antigen cross-presentation by DCs to T cells can be obtained with the so-called “adoptive transfer”. ExAS would be combined with DCs, isolated from the patient’s blood.

These ExAS containing DCs are expected to present the tumor antigen to specific T cells, which after culturing, yield specific cytotoxic T cell clones against the tumor.

Finally, the cytotoxic T cells would be injected to the patient, ready to fight the cancer cells.

Some of the above listed methods are being used in clinical trials and have proven successful in the first phase. It is by no means assured that tumor-derived exosome vaccines without DC injection, will be successful. In fact, an accelerated tumor growth has been observed through NK cell inhibition in mice and in humans as well, in their absence of dendritic cells as carriers for TMPs.

Given their small size, lack of toxicity, target specificity and tolerance in host cells, membrane vesicles may serve as therapeutic agents for the treatment of cancer as well as clinical biomarkers for disease diagnosis and monitoring based on their cancer specific protein, nucleic acid and lipid cargo.

Clinical applications of membrane vesicles are still in the developmental stage and their full potential waits to be explored.

Elucidation of the pathological role of TMPs in cancer is ongoing. Understanding this role is critical in the development of interventions to prevent the progression of cancer, as well as in harnessing this natural mechanism and using it in clinical practice.

In this way, there are three avenues by which TMPs are being studied for improved clinical outcomes.

Firstly, through the formulation of TMP inhibitors as a novel therapeutic class in the treatment of numerous conditions arising from TMP release and the TMP-mediated intercellular communication.

Secondly, harnessing TMPs as natural vehicles in drug delivery.
Utilizing circulating TMPs as cancer biomarkers providing an effective and non-invasive form of cancer diagnosis, prognosis and surveillance to tailor and personalize therapy. These strategies highlight TMPs as attractive therapeutic candidates in disease state management.

TMPs represent a potentially rich source of information obtainable from body fluids through non-invasive approaches. Filtering and interpreting the meaning of this information is challenging.
3.6 CONCLUSIONS

TMPs have particular features and contain active cargo that define their biological role. Indeed, they are involved in intracellular communication and modulation of the microenvironment; moreover, they are characterized by specific abilities as procoagulant and fibrinolytic activities.

In addiction TMPs may be considered as tumor biomarkers and they could help to achieve diagnosis, to predict the prognosis, and to monitor the disease.

Cancer microparticles play also an important role in tumorigenesis and are involved in tumor progression, metastasis, angiogenesis, and escape from immune surveillance and drugs.

It is important to improve their knowledge, because they could be used to define potential new therapeutic approaches.

These TPMS have been isolated in different tumors.

The detection methods of these vesicles were allowed to analyze and better understand them. For these reason, the methods of measurement and standardization of these MPs are needed.
FOURTH CHAPTER: METHODS OF MEASUREMENT AND STANDARDIZATION OF MICROPARTICLES

4.1 PREANALYTICAL VARIABLES INFLUENCING THE MICROPARTICLES MEASUREMENTS

The recent identification of MPs as biomarkers in human pathology explains the variability in analytical conditions described in the literature.

Nevertheless, the growing interest in their detection in pathological situation led, in the 2000s, the scientific subcommittees of Vascular Biology (SSC-VB) of the international Society of thrombosis and haemostasis (ISTH) to establish a standardization work.

The missions are to provide information on technological innovations, to make recommendations in terms of standardization, and to develop calibrators for performing multicentre studies on the measurement of MPs.

The important concept in the analysis of circulating MPs is to control the maximum ex vivo cell activation, to avoid artefactual generation of MPs, while preserving interesting MPs in the sample. The preanalytical phase is, therefore, of major importance in the quality and reliability of results. Indeed, many factors are potential sources of variability (Figure 35). Thus, the work in the subcommittee showed that several factors influence the measurement of MPs: the choice of anticoagulant, the nature of the sampling tube, the sampling mode, the size of the needle used, the transport, the time between sampling and the first centrifugation, the number, the speeds and centrifugation times, the freezing with its specific temperature, the duration, and the thawing method.
This needs the standardization of pre-analytic steps. Both the ISTH and ISEV recently described recommendations for standardizing collection and handling of samples. The recommendations are summarized in Table 12 and consisted on performing the venepuncture with a large needle (21-gauge minimum) and rejecting the first few millilitres of blood taken to overcome the impact of induced vascular injury venepuncture. Plastic tubes seem less activate platelets than glass tubes. The citrate anticoagulation is preferred for minimal cell activation. However, a recent study conducted by György et al. recommended the use of acid-citrate-dextrose (ACD) for the study of MPs, as the ACD blocks in vitro vesiculation process and it does not interfere with the analysis protein or RNA.

The samples must be transported carefully, preferably in vertical position to limit any agitation of the tubes.

The time between sampling and the first centrifugation should not exceed two hours since, beyond this time, the rate of MPs increases, and in particular the platelet subpopulation.
which may be the cause of an artefactual generation of MPs. For this purpose, it is recommended to practice two successive centrifugations at 2500g for 15 min at room temperature, to obtain a "platelet free plasma" (PFP) containing the MPs, which will be stored at -80 °C for offline analysis.

It is important to emphasize that, although the MPs are present in all biological fluids, these recommendations were checked for human blood only. Moreover, these recommendations have been accepted for certain methods of analysis only (CMF, CAT, STA *-procoag-PPL), and only for MPs.

<table>
<thead>
<tr>
<th>Pre-analytical parameters</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube nature</td>
<td>Citrated tubes</td>
</tr>
<tr>
<td>Blood sample</td>
<td>Wide enough needle (21 G minimum)</td>
</tr>
<tr>
<td></td>
<td>Reject the first milliliters of blood</td>
</tr>
<tr>
<td>Delay before first centrifugation</td>
<td>≤ 2 hours</td>
</tr>
<tr>
<td>Transport of tubes</td>
<td>Device holding tubes in vertical position</td>
</tr>
<tr>
<td>Centrifugation protocol</td>
<td>Two following centrifugations: 2500g 15min at room temperature</td>
</tr>
<tr>
<td>Conservation of PFP</td>
<td>-80°C and rapid defrosting in a water-bath</td>
</tr>
</tbody>
</table>

Table 12: Pre-analytical protocol recommended for the analysis of circulating microparticles

PFP: platelet free plasma
(Adapted from Lacroix et al J Thromb Haemost.2012.)

Currently, centrifugation techniques are still the most widely used for purifying PMs. However, the size exclusion chromatography (CET) seems an interesting technique with several advantages. With this method, MPs can be easily separated from the proteins and HDL in the plasma. The separation principle is based on the difference in size. Preliminary data obtained in A. Nieuwland team have proved the value of this methodology for the purification of MPs. The sepharose beads used in column have pore diameter of about 75nm. An MP who enters the ball is delayed because of the path it travels before exiting the column. All particles larger than 75nm, including lipoproteins, cannot enter the ball. The smallest particles present in the fraction 9-12 have a diameter of 70 nm or more.827

With the ETC, on the contrary to centrifugation, there is no risk of protein complex formation
or aggregation of MPs. In addition, the high viscosity of the plasma affects the yield of purification by centrifugation, while it does not affect that of the CET. Osmolarity buffers and physiological density may be used, contrarily to the ultracentrifugation. On the other hand, some teams have reported a loss of biological functions for MPs isolated by sucrose density gradient (ISEV, Budapest, 2013)\textsuperscript{827}, whereas saliva MPs purified by CET remain fully functional\textsuperscript{827}. The MPs purification performance by CET is of the order of 43\% +/− 23, which is equivalent to the purification yields by ultracentrifugation\textsuperscript{828}.
4.2 METHODOLOGIES AVAILABLE FOR MEASURING MICROPARTICLES

The clinical and biological relevance of MPs is now widely recognized; however, quantification and determination of the cellular origin of MPs is still the subject of controversies. This is first due to the complexity of the composition of body fluids and by the difficulty of separating particles of similar size. Different methodologies, based on the characteristics of MPs, have been developed. However, because of their limitations and their practicality, measurement methodologies are not all adapted to the study of biological samples. In the next part, the principle and characteristics of the various available methods will be summarized, giving the advantages and limitations for each of them.

4.2.1 The atomic force microscopy (AFM)

The atomic force microscopy (AFM) is a technique that was developed in 1986\(^{829}\). It belongs to the family of scanning probe microscopes, capable of producing 3-dimensional images. Yuana et al proposed it as a method to study the size, composition and antigenic count subtypes of MPs\(^{830}\). In this technique, the MPs are captured by probes immobilized on a perfectly smooth surface of mica, because it is easy to achieve with this material a clean, flat surface at the atomic scale. Moving a microprobe on this surface can record an electrical signal proportional to the "accidents" of facing surface (corresponding to MPs) (figure 36). Given its resolution of a few nanometres, AFM allows the detection and the accurate count of vesicles in a range of 10nm to 475nm. The size of the PMs can be automatically determined by the interpretation of the height measured by the probe and the volume occupied on the mica surface. This highly sensitive microscopy technique provides precise information on MPs small sizes. Yuana et al showed that AFM can detect 1,000 times more than the MP-CD41 flow cytometry. However, the AFM remains a very heavy machinery to use routinely in terms of the instrumentation and use. In its current form, it is not suitable for large-scale use because of its slow implementation (a few samples per day); it is also limited by its lack of specificity. On the other hand, the AFM needs the immobilization of the sample for the analysis on a flat surface, a difficult step to be controlled in the context of MPs. Finally, the forces applied by
the tip to soft samples, such as MPs, induces a systematic error on the measurement of authors.

Figure 36: Schematic representation of an atomic force microscope
(From geobacter.org)

4.2.2 Light scattering techniques
The Dynamic Light Scattering (DLS), also known as quasi-elastic light scattering, is a non-destructive spectroscopic technique. It determines the size and concentration of MPs suspended in a liquid, by measuring the scattered light intensity fluctuations and the application of a mathematical model of connected Brownian motion of MPs (Stokes-Einstein). It can detect in a sample the submicron particles of size from 1 nm to 6μm. Two instruments based on the principle of DLS were marketed: Zetasizer Nano S, Malvern Instruments Ltd., and N5 submicron particle Size Analyzer from Beckman Coulter. The cellular origin can be determined, by using suitable fluorescent probes as in the "enhanced microscopy laser tracking" (ELMT). This technique has been adapted to track individual particles by Harrison et al: the "single particle tracking" (SPT).

The DLS technique is well suited to the theoretical size range of MPs. However, in its standard configuration, this methodology seems more suitable to the determination of the homogeneous population size. The heterogeneous size distribution of the plasma MPs in biological fluids often leads to uninterpretable results. This technique measures all types of particles in a size of analysis window that may include artefacts. Indeed, the presence of larger particles, lipoprotein, aggregates or other sources of contamination diffuses more light than the smaller
particles and will have the effect of moving the obtained size to higher values. The use of fluorescent probes in derivative techniques such as SPT or ELMT was introduced to overcome such limit.

The Nanoparticle Tracking Analysis (NTA) is a recent technology, based on light diffusion, for visualizing and analysing the suspended particles in real time; it is marketed under the name Nanosight® (Amesbury, United Kingdom). This is a technique of videomicroscopy, also based on the Brownian motion of particles in suspension, but, unlike DLS, it records the individual motion of each particle in suspension. The instrument uses a laser, which passes through a suspension of particles through a glass prism. The laser beam of light that diffuses allows visualizing the particles that correspond to points of light. A video of the movements of the particles is recorded and analysed by the software in NTA, which is then able to identify each particle individually by its Brownian motion. The equation of Stokes-Einstein is applied to determine the size of each particle. This technique allows to analyse theoretically vesicles of diameter between 50 nm and 1 um. It is compatible with the use of fluorochromes, and therefore allows phenotyping analysed MPs.

However, the NTA is less precise on heterogeneous samples; indeed, one of its limitations is its moderate ability to differentiate particles with near size. Indeed, two populations of vesicles can be differentiated only if their size differs by more than 1.5 times. Moreover, in biological samples, the measurement of the concentration of vesicles of different diameter is strongly influenced by the size and the refractive index of the vesicles. The use of calibration silicone beads with known size and concentration, and optimization of the gain of the camera, should allow to correct this inconvenient. In its current form, the NTA is a time-consuming technique that requires considerable expertise, which limits for now its use in analytical laboratories.
4.2.3 Resistive pulse sensing (RPS)

A new technique, the Resistive pulse sensing (RPS), can detect accurately (in theory) the size of the vesicles in suspension whose diameter is between 70 nm-10 μm, based on the variations of impedance (Coulter principle)\(^{838}\). A new instrument was developed and marketed, the qNano (Izon Science Ltd, Christchurch, New Zealand) (Figure 38). The instrument consists of two fluid cells divided by a membrane having a pore. Each cell is immersed in an electrode to allow a current to flow through the pore, generally less than 1 μm in diameter. The vesicles in suspension are driven through the nanopore membrane and each passage generates a variation in the signal, which is recorded. Signal magnitude is proportional to the size of the particle and the recording frequency of these signals is directly proportional to the concentration of the sample measurement\(^{839}\). Several pore diameters are available, which allows extending the vesicle size-measuring interval.

This technology seems well suited to the measurement of small MPs. However, its use in biological samples is still limited by its lack of specificity and the risk of clogging the filter. Indeed, one of the principal limitations lies in the frequent obstruction of the nanopore by the accumulation of high molecular weight molecules (fibrinogen, vWF, apoptotic bodies, small...
cells, proteins, vesicles or calibration beads aggregates ...). Clogging the pore and the presence of contaminants in the sample can affect the measurement sensitivity. The sample preparation must eliminate large vesicles and reduce the concentration of protein. Moreover, regular calibration is recommended. Because of this limit, the measurement time for biological sample varies from 30 minutes to 1 h.840 For this reason, the qNano is considered a research tool rather than a tool suitable for routine clinical analysis. To overcome this drawback, Izon Science is currently developing an automatic cleaning system ultrasonic pore, but it will require careful development because the sonication may artificially create new MPs in the sample.

![Figure 38: Schematic representation of the resistive pulse sensing (RPS).](image)

A. Two fluid cells, with an immersed electrode, are separated by a nanopore.
B. The current \( I \) is measured by time, \( \Delta I \) variations correspond to the successive passage of two vesicles (From Van der Pol and extract al.J Thromb Haemost.2013).

### 4.2.4 Innovative techniques

- The cryo-electron microscopy (cryo-EM) is an electronic microscopy technique developed by the team of A. Brisson for analysing MPs. It allows visualizing the size, morphology and phenotype of MPs by gold nanoparticles functionalized by Annexin V or specific antibodies841. This is a very sensitive but difficult technique to implement (Figure 39).

- The **Nuclear Magnetic Resonance (NMR)** is a new technique which allows sensitive, rapid and accurate MPs analysis in biological samples. It requires a marking of MPs by an antibody, coupled to magnetic nanoparticles. Miniaturization of the nuclear magnetic resonance system (μRMN) measures the magnetic difference between the biological sample and the MPs. This
technique requires only a small amount of blood; it is easy and cheap to use. It has been recently used to measure bloody MPs in transfusion bags and in a clinical study for the measurement of glioblastoma MPs to evaluate the effectiveness of treatment\textsuperscript{607}.

\textbf{4.2.5 Flow cytometry (FCM)}

Because of its accessibility and versatility, flow cytometry is to date the most widely used technique for the measurement and characterization of MPs in biological samples\textsuperscript{842, 843, 844, 845}. It allows a high throughput analysis of cells and particles in suspension in a liquid flow, causing them to flow towards an analysis area (flow cell) where they interact individually with a laser beam. Detecting the light scattering and the emitted fluorescence enables simultaneous analysis of the physical characteristics and the antigenic composition, with over 1000 events per second. Indeed, flow cytometry is a quantitative technique that not only allows for absolute counting of MPs, based on the determination of size (<1 um) and the expression of PS revealed by labelling with the annexin V, but it also determines their cellular origin by the detection of surface markers revealed by specific antibody (Table 13)\textsuperscript{846}. This increases the multiparametric analysis of MPs detection specificity. In addition, the speed of implementation of this technique contrasts with sensitive but time-microscopy techniques. It is also possible to obtain an absolute count in a wide range, using counting beads of known concentration.
<table>
<thead>
<tr>
<th>MP</th>
<th>Original Cell</th>
<th>Antigen of membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPP</td>
<td>Platelets</td>
<td>CD31, CD40L, CD41a, CD42a, CD42b, CD61, CD62P, CD63, CD107a, Fibrinogen, FvW</td>
</tr>
<tr>
<td>MPMeg</td>
<td>Megacaryocyte</td>
<td>CD41, CD184, filamine A</td>
</tr>
<tr>
<td>MPLy</td>
<td>Lymphocyte</td>
<td>CD3, CD4, CD8, CD11b, CD16, CD19, CD20, CD14, CD45, CD66b</td>
</tr>
<tr>
<td>MPMo</td>
<td>Monocyte</td>
<td>CD14, CD142, CD144, CD162</td>
</tr>
<tr>
<td>MPery</td>
<td>Erythrocyte</td>
<td>CD35, CD235a, IgG, complement</td>
</tr>
<tr>
<td>MPPNN</td>
<td>Polymorphonuclear</td>
<td>CD11a, CD11b, CD15, CD16, CD35, CD49, CD59, CD62L, CD66b, CD142</td>
</tr>
<tr>
<td></td>
<td>neutrophil</td>
<td></td>
</tr>
<tr>
<td>MPE</td>
<td>Endothelial cell</td>
<td>CD31, CD34, CD51, CD54, CD62E, CD63, CD105, CD106, CD142, CD144, CD146</td>
</tr>
</tbody>
</table>

**Table 13: Surface Markers of vascular and hematopoietic PMs depending on the transmitting cell**


However, it has several limits: the difficulty in detecting events with low antigen expression, an expensive count of MPs and the difficulty in detecting small events (<0.2μm).

Cytometers used until now measure only part of the MPs (large cells). Indeed, the MPs are vesicles of small and heterogeneous size between 0.1 to 1. um, according to electron microscopy. The currently available cytometers are unable to count all MPs, except the largest. However, it is hard to estimate the smallest size detectable by MPs cytometer. The size of the calibration beads is not exactly equivalent to the size of MPs, due to a difference in the refraction index. Consequently, a cytometer capable of detecting small latex beads does not imply that it can detect similar sized MPs. In addition, the performance of the instruments
varies between types of machines, and sometimes a same instrument of the same type and the same device can vary in time. However, it is estimated that the smallest detectable MP is around 400nm to 200nm by a standard cytometer with high sensitivity. Depending on the antigen density on the MP and the ratio between signal and background noise, events lower than 200 nm can be detected by fluorescence. MPs numeration limits depend on both the fluorescence sensitivity of the instrument and its intrinsic resolution limit to discriminate events determined from the background noise. It is typically described that the most used wavelength (488nm) is the major determinant of detection limit, however, despite its impact in the light scattering physics, numerous studies have shown that this assertion is not entirely correct. Out of the potential sources of background noise of the instrument that disturb the detection of MPs, the optical noise has usually the greatest impact. Thus, optimization could greatly improve the MPs count. Because the forward scatter conditions cytometers (FSC) must be measured in a narrow angle range close to a very intense light source (the laser beam itself), a very accurate alignment of the laser is necessary for measurements of fluorescence or light scattering. The positioning of the obscuration bar directly blocking the laser light is also critical. Therefore, it is recommended to realize the laser alignment by an experienced technician, especially for essays with MPs. Optical noise can also come from the presence of dust particles on the analysis cell; this noise increases gradually with time. It can be reduced by working in a protected atmosphere and by regular cleaning of the analysed cell.

We are, therefore, faced with an iceberg where only the small part of the tip is visible, because most interesting MPs are still undetectable. The standardization process is an essential step for the realization of multi-centre clinical studies to confirm the interest of MPs in human pathology. To overcome the current limitations, two main strategies have been proposed: the standardization of the analysis window of MPs in a small and well defined range, and the improvement of MPs detection of small size with the new arrival of high sensitive cytometers. For this purpose, six years ago we proposed a strategy based on the use of calibration latex beads called the "Megamix" (Biocytex, Marseille, France), corresponding to a mixture of fluorescent diameter latex microspheres (500-900nm) and defined ratio. This calibrator is designed to determine the FSC and thus define MPs analysis window to improve the reliability and reproducibility of measurements (Figure 40). This strategy has been tested during an international workshop, bringing together 40 laboratories, corresponding to 59 cytometers. During this work, several laboratories obtained reproducible values of MPPs, particularly with the Beckman Coulter instruments, the FC500 or EPICS XL. However, this strategy has proved
for the Becton-Dickson (BD) cytometers. These differences in the collection of the FSC are at the origin of these gaps, mainly because of the use of different collection angles. However, having found a better resolution and better uniformity in SSC in this subgroup of flow cytometers, a new MPs numbering standardization strategy based on the SSC has been proposed. A new kit of beads for standardization, suitable for instrument using the SSC, has been developed. This new strategy provides MPPs counts that are equivalents between the two subgroups of instruments. From this new protocol in the standardization subcommittee ISTH, a new international multicentre workshop to evaluate this large-scale protocol has been organized.

A new generation of flow cytometers is now available with optical and fluidic characteristics being more suitable for MPs measuring (Apogee A40 / 50, LSRII Fortessa, BD Influx, Gallios / Navios). Significant progress has been made in the small vesicles detection strategy. Indeed, these high sensitivity cytometers perform better in terms of resolution and have a lower noise floor, enabling them to detect 8 to 20 times more MPs compared with standard cytometers. Most of these instruments are equipped with high performance photomultipliers, instead of photodiodes, for the detection of the FSC signal. The FSC collection angle is enlarged, increasing the sensitivity and detection of small MPs. Remarkably, the flow cytometer BD influx can detect 100 nm particles with sufficient resolution to distinguish easily 200 nm particles based on the diffused light. The measurement of MPs on Apogee A50, of plasma of healthy subjects in preanalytical ideal conditions increases the rate of MPPs annexin V + 100 / μl to 1370 / μl. This improvement was also observed with other subtypes of MPs: MPerys, MPLS, MPEs. With settings defined on the BD influx, an optimized labelling protocol and a fluorescence threshold set, this technique is useful for the detection and characterization of nanoscale vesicles derived from in vitro cell culture. The ratio of small and large MPs may vary, depending on the clinic and the subtype MPs. Little is known about the differences in properties and nature of these large and small MPs detected by CMF. Several assumptions have been made regarding the big MPs: small platelet aggregates or even a cluster of small MPs. The recent availability of these small MPs opens many questions; these new techniques still need to be optimized, especially for the low intensity fluorescent labelling of smaller MPs. It interesting to note that a new standardized tool was developed to be adapted to these small MPs (Megamix more Biocytex).
Figure 40: Schematic representation of a flow cytometry.
Graphs cytometry bead on Navios Megamix (Beckman Coulter, CA, USA) FS threshold: 0.3μm. (personal data). The sample is driven by a sheath liquid to an analysis cell, each vesicle interacts with the laser causing light scattering face (FSC: forward scatter) and 90° (Side scatter and fluorescence). The light signals and fluorescence are collected and analyzed (From Van der Pol and al. J Thromb Haemost. 2013).

4.2.6 Functional tests
The second category of methods is composed of a set of techniques providing information on the activities of MPs.

- Capture Tests
They are based on the combination of a capture test and a functional test. They use either Annexin V coupled to a solid support that capture the MPs expressing PS, or antibodies which recognize specific antigens expressed by MPs. MPs are thus measured based on their cellular origin and/or their content in PS. For example, the test Zymuphen MP-Activity® (Fig. 41), marketed by Hyphen Biomed (Neuville-sur-Oise, France), combines a solid phase capture of MPs by Annexin V or an antibody and measure the prothrombinasic activity of MPs. These tests have the advantage of achieving high-speed analysis of many samples. They are sensitive enough to detect weakly present antigens (Zymuphen MP-Activity has a detection limit ≤ 0.05nM in PS equivalent to a plasma normal <5 nM). These tests are reproducible, widely validated and easy to perform in the lab. However, they provide no information on the size of the measured absolute MPs or account. An indirect quantification is possible; however, the measured signal can be converted into the equivalent concentration using a solution of liposomes of composition and known size \(^{857}\). However, this conversion means that MPs behave strictly as liposomes, which remains a difficult hypothesis to be confirmed. The overall
quantification depends on a single measured biomarker. MPs having heterogeneous sizes, it is also possible that capturing PMs differs depending on the diameter of the MPs. Interference with soluble antigens are possible and may lead to overestimation levels MPs. Functional tests measure the overall contribution of MPs to a specific activity, not necessarily related to their number. This is particularly true when minority subpopulations have the highest activity.

Figure 41: Principle of a hybrid method combining capture and functional test. MPs are captured by antibodies previously fixed on a plastic surface. After that, MPs are placed in presence of clotting factors Va and Xa and prothrombin. The formed thrombin is revealed by a chromogenic substrate spectrophotometry. The amount of formed thrombin is proportional to the amount of PMs in the sample. TF: tissue factor; AV: Annexin V; BI: Biotin; SA: Streptavidin; PS: Phosphatidylserine. (Zymuphen * Hyphen Biomed). (According to JM. Freyssinet).

- Thrombin Generation Test (TGT)
The generation of thrombin dependent MPs can be performed on the Calibrated Automated microplate Thrombogram® system (CAT) (Thrombinoscope BV, Maastricht, NL and Diagnostica Stago, Asnieres, France).
It is a semi-automatic test that analyses the thrombin activity in the clotting, including the inhibitory systems, without taking into account primary hemostasis and the final part of the network structure of fibrine. The thrombin formed is visualized using a fluorescent substrate.
The thrombin generation curve was determined after correction by a calibrator, it is characterized by a lag phase followed by the formation and subsequent inhibition of thrombin formation. The area under the curve allows determining the thrombin concentration in the sample (in nm/l). Depending on the reagents used, dependent activity of the phospholipids of MPs (MP-reagent) or FT-dependent activity of MPs (PRP-reagent) (Diagnostica Stago) can be determined. However, one of the major problems of this test remains the difficulty of standardization.

![Thrombin generation curve](image)

**Figure 42: Plasmin generation curve obtained from the CAT**
FTE: area under the curve; Lag time: latency, peak height: peak height. 
*(From Castoldi and al. Thromb Res. 2011)*

- **Clotting Time phospholipid-dependent**: STA®-Procoag-PPL (Stago, Paris, France)
This test is based on the principle of Factor Xa based clotting time assay (XACT), previously described by Exner et al in 2003.

It measures the procoagulant activity of the MPs dependent phospholipids. In this test, MPs are suspended in a plasma without of MPs (Microparticle Free Plasma = MPFP), the triggering reagent coagulation (FXa + Calcium) is without phospholipids, which makes the test phospholipid-dependent made by MPs. The clotting time measured is inversely proportional to the power coagulant dependent phospholipid component MPs. This is a very reproducible and automated test as it is feasible in routine laboratory coagulation PLC.

- **Factor Xa generation test** (measuring the activity of the MPs dependent FT)
The principle is the following: MPs pelleted by ultracentrifugation, are brought into contact with an anti-TF monoclonal inhibitory antibody or isotype antibody. The addition of a reaction mixture, containing FVIIa and FX, is used to trigger the coagulation cascade. FXa generated is measured using a chromogenic substrate. The amount of FT is determined using a calibration curve.

An adaptation of this test with improved sensitivity has recently been proposed by Biocytex. Standardization of this test is essential and requires inter-laboratory comparison.

<table>
<thead>
<tr>
<th>Method</th>
<th>Quantification</th>
<th>Phenotyping</th>
<th>Size</th>
<th>Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron microscopy</td>
<td>Limited</td>
<td>Limited</td>
<td>Yes</td>
<td>Artefacts during sample preparation</td>
</tr>
<tr>
<td>Functional tests</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Accessibility of the PS or the active molecule carried by the MPs</td>
</tr>
<tr>
<td>Atomic force microscopy (AFM)</td>
<td>Limited</td>
<td>Limited</td>
<td>Yes</td>
<td>Artefacts (cellular debris and proteins)</td>
</tr>
<tr>
<td>Dispersion of lights (DLS or NTA)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Artefacts (cellular debris and proteins)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Accessibility of the PS or the active molecule carried by the MPs</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>No</td>
<td>Oui (extraction des protéines)</td>
<td>Non</td>
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<td>Yes</td>
<td>Limited</td>
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<td>Cytometry imaging</td>
<td>Yes</td>
<td>Yes</td>
<td>Limited</td>
<td>Fluorescence of MPs</td>
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<tr>
<td>Resistive pulse sensing (RPS)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Artefacts (cellular debris, proteins and lipids)</td>
</tr>
</tbody>
</table>

Table 14: Technics of MPs measurements.
Currently, none of these techniques meet all the desired criteria for measurement of plasma MPs. Because of the absence of an ideal method, this diversity must be understood and used in a sense of complementarity. A direct comparison between these techniques is needed to objectively determine the relative contribution of each of them. In addition, the realization of multi-center clinical studies to confirm the interest of MPs in human disease makes essential a standardization of these methodologies. The goal is to develop a measurement method that is sensitive, specific, reproducible, standardizable, low cost, applicable to large series and automated to be transferred in non-specialized laboratory.
SECOND PART: PERSONAL WORK
CHAPTER 1: DEFINITION OF WORKS

1. THE PROJECT

1.1.1 Scientific background/Rationale
Malignant pleurisy is very common in patients with cancer\textsuperscript{865 866} reflecting the dissemination of malignancy and the advance disease\textsuperscript{867}.
Currently, the differential diagnosis is based on mini-invasive approaches such as thoracoscopy with a high diagnostic yield. However, non invasive biomarkers that can discriminate between benign and malign pleurisy are needed in particular during the patient’s follow-up.
Among possible non invasive markers, microparticles (MPs) are extracellular vesicles which result from the blebbing of cell membrane in response to activation or apoptosis, and are released by all eukaryotic cells, including cancer cells\textsuperscript{344 408}.
They are generally characterized by a size comprised between 0, 1 and 1 micron, the expression of the anionic phospholipid phosphatidylserine (PS) membrane antigens representative of their parental cells so that their origin can be determined by using specific antibodies and annexin V\textsuperscript{344}. These characteristics distinguish MP from exosomes which are smaller in size, are devoided of PS and result from multivesicular bodies.
The presence of MP and exosomes have been reported in human body fluid including plasma and other liquids such ascites fluid\textsuperscript{300 319 868}.
There are only few data available about extracellular vesicles in pleural liquid\textsuperscript{325 869}.
To our knowledge, this is the first work that directly try to identify tumour derived MPs in pleural liquid from different aetiologies, using specific antibody labelling for neoplastic markers.

1.1.2 Aim
The objectives of this work are to directly identified tumour derived MPs in pleural liquid from different aetiologies, using specific antibody labelling for neoplastic markers.
Therefore, we hypothesized to achieve results than can lead in short term a new diagnostic approach and in long term the possibility to progress based on larger cohorts of patients and on
several studies and it lays the basis of tumoral antigen MPs as potential biomarkers for classified pleural effusion.

1.1.3 Hypothesis

Because tumour cells produce high amount of MPs, we hypothesized that tumour derived MPs could be present in pleural liquid and could help to identify in a non invasive way patient with malignant pleural effusions. Moreover, we hypothesized that the detection of specific MPs might be useful as a complementary tool with the cytology to better discriminate benign from tumoral origin of pleural effusion; as a marker patients’ subpopulation that may benefit from personalized therapeutic approach, and as non invasive monitor of the response to treatment.
1.2 MATERIAL AND METHODS

Patients with pleural effusion were included: case-patients with histologically-proven primary or metastatic pleural cancer and control-patients age-matched patients with cytologically negative pleural effusion without neoplastic cells or cell atypia.

The cytological and histological diagnosis obtained by thoracentesis or thoracoscopy were standardized.\textsuperscript{871,872}

Pleural sample were centrifuged, collected and stored at -80°C until use.

MPs were purified by high speed centrifugation and enumerated by high-sensitivity flow cytometry following standardization.\textsuperscript{849}

We tested several markers and we will measure them by ELISA test (DuoSet ELISA kit-R&D Systems).

Statistical analyses were performed with GraphPad Prism software v.5.0 GraphPad Software, San Diego, CA, US.
1.3 OBJECTIVES

The principal aims of project could be summarized as follow:

- To define protocols for manipulation and stockage pleural samples.
- To verify whether microparticles (MPs) are present in pleural effusions.
- To characterized MPs defining their mother cell: are there tumoral MPs in pleural liquid?
  - To evaluate whether MPs in pleural fluid have some activity.
  - To evaluate diagnostic, prognostic and predictive value of MPs, comparing biological with clinical data.
- To analyzed the correlations between different body fluids from the same patient (pleural fluid, bronchoalveolar lavage, peripheral blood)
  - To assess whether the MPs from pleurisy have a "genetic signature" (microRNA and molecular biology).
- To design therapy protocols against target receptors on the MPs.
1.4 WORKPLAN

1.4.1 Manipulation and stockage of pleural samples
Definition of protocols for manipulation and stockage pleural samples is needed because in literature there are very few data about these procedures. Therefore, it was important to define how pleural fluids can be centrifuged, and for this reason, we tested different methods of centrifugation in order to obtain the optimized protocol. Moreover, we defined a protocol for the storage of collected supernatant and pellet. The definition of these protocols is important to obtain good samples to detect events we are looking for avoiding background noise.

1.4.2 Are microparticles (MPs) present in pleural effusions?
Because few data are available about extracellular vesicles in pleural liquid, we looked for their presence using flow cytometry. In particular, we searched extra cellular vesicles with a size included within a analysis window defined by 0.3 to 0.9 µm beads and positive for annexin-V+ labeling detectable in pleural fluid, both from neoplastic and non neoplastic aetiology. These features were defined as compatible with MPs definition.

1.4.3 Characterization of MPs from pleural liquid
In order to characterize the cellular origin of these MPs, we first performed complementary immunophenotyping with antibodies allowing the measure of erythrocytes (EryMP), platelets (PMP), leukocytes (LMP) and endothelial-derived MPs (EMP). We analyzed the composition in these haematopoietic and vascular MPs subpopulations and we checkd it is significantly different between malign and benign pleural effusions.

1.4.4 Are tumour derived MPs present in malignant pleural effusion?
We tested the presence of selected tumour associated markers on pleura. In particular, at the beginning we checked the presence of these neoplastic markers: podoplanin, EpCAM, and mucin 1.
The expression of podoplanin is upregulated in mesothelioma and others human cancers. On the other hand, podoplanin is also expressed on physiologic mesothelial cells and others normal tissues.

Similarly, MUC1 can be expressed by tumors such as mesothelioma as well as by normal tissues such lung, mammary gland, uterus, esophagus, stomach, duodenum, pancreas, prostate, and hematopoietic cells.

We hypothesized that tumour derived MPs could be present in pleural liquid and could help to identify in a non invasive way patient with malignant pleural effusions.
CHAPTER 2: ARTICLES

2.1. INTRODUCTION

Microparticles (MPs) are submicron extracellular vesicles that result from remodelling of membrane phospholipids in response to cellular activation or apoptosis. They represent both biological effectors with multiple effects on homeostasis of different systems and biomarkers detectable in biological fluids, which are representative of a pathological process or of a pharmacological response to therapeutic intervention. Moreover, MPs can be released from any type of cells: this is very interesting in the field of oncology, because the presence of microvesicles released by tumor cells was demonstrated and it could be correlated with neoplastic pathogenesis. Tumor microparticles (TMPs) are an attractive object of study, because they might become specific biomarkers, easily accessible by non-invasive samples and they could be a tool for monitoring the disease as well as a new therapeutic targets.

My PhD thesis is developed in this context and, in particular, it aims to check for the first time cancer MPs present in the pleural fluid. This work is divided in two different parts. The first one describes MPs and their characteristics, and allows to understand the role of TMPs in cancer pathogenesis. The second part is about my personal work. We hypothesised that MPs were present in pleural fluid. If this hypothesis was confirmed we tried to characterize the MPs detected from pleurisies, defining their parental cells. Moreover, we thought that MPE could contain TMPs and we planned to investigate their role in early diagnosis and in monitoring of disease, and their specific activities eventually involved in cancer pathogenesis.

Our first work aim at directly identify TMPs in pleural liquids. Indeed, we demonstrated, for the first time, the presence of a large amount of MPs in pleural effusions originating from a large panel of normal and malignant cells. We defined the cellular origin of MPs, and we showed for the first time the presence of tumor-derived-MPs in this biological compartment.
In addition, using a specific labelling, we detected EpCAM-positive-MPs (EpCAM+ MPs) from pleural liquid. These TMPs seem to be related to adenocarcinoma histology. (Article 1). This first project was also selected as an oral presentation at the European Respiratory Society Congress 2015 International Congress (Annexe 1).

Moreover, we identify patients suffering from cancer, characterized by the detection of EpCAM-MPs in pleural fluid, but with negative cytology for malignant cells (Article 2). Therefore, this finding would help the oncologists to early achieve diagnosis. Indeed, EpCAM-MPs could be considered a complementary tool to cytological routinely analysis. Moreover, these MPs could be useful to identify the subpopulation of patients that may benefit from earlier therapeutic approach, for example against EpCAM antigen.

The feasibility of detecting tissue factor (TF) expressing MPs by flow cytometry (FCM) is a matter of debate. With tumor-derived MPs in these fluids, both a high MP-associated TF-specific procoagulant activity (MP-TF PCA) and TF+ MPs (MP-TF) may be expected. Therefore, in a subsequent work, we proved that MP-TF PCA may complement the immunological detection of EpCAM+ MPs for mini-invasive identification of patients with malignant pleural effusions (Annexe 2).

This thesis could open the way to evaluate diagnostic, prognostic and predictive value of MPs, and to define the origin of metastatic pleural disease using specific tumoral antigen MPs against the primitive cancer (ErB1, PSA, CA19.9, CA15.3, CA125…). Moreover, our work could open new perspectives to study MPs activities and impact in the pleural microenvironment.

Finally, our data could be useful to assess whether the MPs from pleurisy have a "genetic signature" (microRNA and molecular biology), and to design therapy protocols against target receptors on the MPs.
2.2. ARTICLES

ARTICLE 1

Pleural biomarkers allowing to mini-invasively discriminate benign from malignant pleural effusions are needed. Among potential candidates, MPs are extracellular vesicles that vectorise antigen derived from the parent cell. We hypothesized that tumor-derived MPs could be present in the pleural liquid and help to identify patients with malignant pleural effusions. Using highly sensitive flow cytometry and cryo-electron microscopy, we showed that large amounts of MPs from hematopoietic and vascular origin could be detectable in pleural fluids. Their level did not differ between benign and malignant pleural effusions. Analysis of selected tumoral associated antigens evidenced for the first time the presence of tumor-derived MPs expressing EpCAM in malignant pleural fluids only. The detection of EpCAM-positive-mps (EpCAM+ MPs) by flow cytometry showed a better specificity and sensitivity than ELISA to distinguish between pleural carcinoma and the others malignant pleural effusions. Combining EpCAM+ MPs and cytology improved the diagnosis of MPE compared to cytology alone. This study establishes the basis for using EpCAM+ MPs as a promising new biomarker that could be added to the armamentarium to mini-invasively identify patients with malignant pleural effusions.
Detection of EpCAM-positive microparticles in pleural fluid: A new approach to mini-invasively identify patients with malignant pleural effusions

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*These authors contributed equally to this work


INTRODUCTION

Malignant pleural effusion (MPE) is very common in cancer patients [1, 2] reflecting the dissemination of malignancy as well as advanced disease [3]. The differential diagnosis is based on invasive approaches such as thoracoscopy which has a high diagnostic yield and represents the gold-standard at the present time. Pleural biomarkers which could discriminate benign and malignant pleural effusion are needed for the diagnosis but also for monitoring during the patient’s follow-up. Among potential candidate biomarkers, microparticles (MPs) are Extracellular Vesicles (EVs) released by all eukaryotic cells including cancer cells [4]. MPs result from the blebbing of cell membranes in response to activation or apoptosis and vectorize antigens from their parent cells. MPs are characterized by size, ranging from 0.1 and 1 micron. They generally express the anionic phospholipid phosphatidylserine (PS) and membrane antigens representative of their parental cells [5]. These characteristics distinguish MPs from exosomes which are smaller in size, devoid of PS and originate from multivesicular bodies.

The presence of MPs and exosomes has been reported in human body fluids including plasma and other liquids such as bronchoalveolar liquid, urine and ascites fluid [6–9]. To our knowledge, little is known about the presence of MPs in pleural fluid [10, 11]. Because tumor cells produce high numbers of MPs [12], we hypothesized that tumor-derived MPs could be present in pleural fluid and help to mini-invasively discriminate benign from MPE.
RESULTS

We investigated the presence of MPs in pleural fluids using highly sensitive flow cytometry. As shown in Figure 1, extracellular vesicles with light scatter properties compatible with those of MPs (Figure 1A and 1B) and positive for Ann-V+ (Figure 1C) were detectable in pleural fluids of neoplastic and non-neoplastic etiologies (n = 85). Additionally, Cryo-Transmission Electron Microscopy analysis (cryo-TEM; Figure 1D) confirmed the presence of extracellular vesicles with sizes ranging from 0.1 and 0.5 μm. These features were compatible with MP definition. Using Ann-V labeling, no significant differences in the total MP count were found between benign and MPE (3500 MP/μL [2400–7800] vs 7300 MP/μL [3200–11000], respectively; p = 0.18) (Figure 1E). To characterize the cellular origin of these MPs, we first performed complementary immunophenotyping with antibodies specific for erythrocyte-(EryMP), platelet- (PMP), leukocyte- (LMP) and endothelial-derived MPs (EMP). As illustrated in Figure 1F, the level of MPs from hematopoietic and vascular origin which does not differ between benign and MPE. We concluded that MPs from hematopoietic and vascular origins failed to discriminate benign from malignant pleural effusions. These results are in agreement with the notions that inflammation and vascular activation are common features of pleurisies regardless of origin.

In both malignant and benign pleural effusions, the detection of about 70% of the Ann-V+MPs (4480+/− 4830 MP/μL) that fail to express vascular or hematopoietic markers, prompted us to investigate the presence of selected tumor-associated markers. MPE can be divided into primary pleural cancer (malignant pleural mesothelioma) or secondary pleural metastases from other neoplasia (lung, breast, prostate...). Among metastatic pleural cancers, lung cancer is the most frequent etiology. Therefore, we analyzed the most common immunohistochemical markers used in the differential diagnosis between epithelioid pleural mesothelioma and lung adenocarcinoma [17]. Among them, we choose surface markers which are present at the cell membrane and therefore potentially present at the MP surface: podoplanin, mucin 1 and EpCAM. Podoplanin+MPs and mucin 1+MPs were found in pleural effusions of both cancer and benign origin (Figure 2A and 2B). Therefore both podoplanin and mucin 1+ failed to assign the malignant etiology of pleural fluid. This is consistent with the expression pattern of podoplanin found to be upregulated in mesothelioma and other human cancers [18, 19]. However, podoplanin is also expressed in mesothelial cells and other normal tissues [20, 21]. Similarly, mucin 1 can be expressed in tumoral and normal tissues including lung, mammary
gland, uterus, esophagus, stomach, duodenum, pancreas, prostate, and hematopoietic cells [22, 23].

By contrast, significant amounts of AnnV+ EpCAM+ events were detected in malignant pleural effusions only (Figure 2C). To investigate whether these events could be specific for EpCAM, an immunomagnetic depletion (IMS) was performed using beads coated with an anti-EpCAM antibody. After IMS, more than 90% of the Ann-V+/EpCAM+ MPs were removed (Figure 2D) whereas no depletion was observed when IMS was performed with beads coated with an irrelevant antibody. These results demonstrate that flow cytometry can be used to specifically detect EpCAM+ MPs in MPE. Moreover, in order to compare the proportion of EpCAM bound to MPs or released as a soluble form, we used high speed centrifugation to separate the vesicular from the soluble fractions. Interestingly, as illustrated in Figure 2E, we found that most of EpCAM antigen was detectable in the pellet whereas the amount remaining in the supernatant was non-significant. These results demonstrate that the majority of EpCAM detectable in pleural fluid is bound on the MP surface.

Then, the capacity of EpCAM+ MPs to distinguish benign and MPE was evaluated by two methods: 1) high sensitive flow cytometry, performed directly on the pleural fluid and 2) ELISA for EpCAM antigen performed on the MP pellet after high speed centrifugation of the pleural fluid. As shown on Figure 3A, detection of EpCAM+ MPs by flow cytometry was found in 50% (35/70) of cancer patients. In these patients, the median level was 176 [37–665] EpCAM+ MPs/μl, median [25th–75th interquartile range]. Only 1 out of 14 patients with benign pleurisies presented a very low number of EpCAM+ MPs (9 MPs/μl). Using an ELISA test with a detection limit at 1 pg/ml, EpCAM+ MPs were found in 44% (32/71) of cancer patients with a median value of 228 [7–595] pg/ ml (Figure 3B). Only 1 out of 14 patients with benign pleural effusions was positive. Thus, in this cohort of patient, the detection of EpCAM+ MPs showed a good specificity (sp = 93% for flow cytometry and ELISA) and a low sensitivity (Se = 49% and 45% for flow cytometry and ELISA, respectively) to distinguish between benign and MPE.

Because, EpCAM antigen is not expressed by all tumors, we divided malignant patients into two groups according to their etiology: 1) carcinoma (n = 44) known to generally express EpCAM, and 2) non-carcinoma (n = 27, mesothelioma, melanoma, lymphoma and sarcoma) generally not expressing EpCAM. By flow cytometry, EpCAM+ MPs were detected in 79% (34/43) of carcinoma pleurisies with 221 [42–670] EpCAM+ MPs/μl compared to only 1 out of 27 patients with non-carcinoma malignant pleurisies with a very low number of EpCAM+ MPs (11 MPs/μl) (Figure 3A). In contrast, using ELISA test, the detection of EpCAM+ MPs
was positive in 66% (29/44) of carcinoma pleurisies with 233 [42–713] pg/ml but also in 3/27 with non-carcinoma malignant pleural effusions (mesothelioma) with a low concentration EpCAM (7 pg/ml) (Figure 3B). Thus, the detection of EpCAM+ MPs by flow cytometry showed a better specificity and sensitivity than by ELISA (Sp: 96% vs 89%; Se: 79% vs 66%) to distinguish between carcinoma and non-carcinoma MPE. Consequently flow cytometry was selected to measure EpCAM+ MPs in the next part of the study.

Among patients diagnosed as carcinoma, we therefore compared tumor cell detection by cytology (the reference method) to EpCAM+ MPs enumeration by flow cytometry, for their capacity of to differentiate benign and MPE (Table 1). Patients were stratified according to the presence of EpCAM+ MPs. A perfect agreement between tumoral cells and MPs was found for 68% of patients.

Interestingly, in 3 cases, only EpCAM+ MPs were detected. Conversely, cytology alone was positive in 5 cases for which, 3 pleural fluids presented less than 5%

Figure 1: Microparticles in pleural effusions.
A. Flow cytometry scattergram of the microparticle (MP) window of analysis determined by FSC-Megamix-Plus beads. B. Representative scattergram of the pleural fluid events appearing in the MP window. C. Representative dot plot showing the annexin-V (AnnV) positivity of the pleural fluid extracellular vesicles. The control experiment was performed in the presence of phosphate buffered saline buffer (PBS) compared to Ca2+-containing binding buffer (BB). D. Representative image of pleural fluid extracellular vesicles by cryo-transmission electron microscopy. E. Total MP counts by flow cytometry (TMP = AnnV+MPs) between benign B. and cancer C. pleural fluids. F. Hematopoietic and vascular MP subpopulation enumeration by flow cytometry between benign B. and cancer C. pleural effusions. Platelet-derived MPs (PMPs): AnnV+/CD41+; erythrocyte-derived MPs (Ery-MPs): AnnV+/CD235a+; Leucocyte-derived MPs (Leu-MPs): AnnV+/CD11b+; endothelial-derived MPs (EMPs): AnnV+/CD41−/CD31+. NS = no significant difference.

Table 1: EpCAM-positive MP detection and cytology in pleural effusions from primitive carcinoma
<table>
<thead>
<tr>
<th>DIAGNOSIS</th>
<th>EpCAM + MPs (FCM)</th>
<th>Malignant cells (Cytology)</th>
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</thead>
<tbody>
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<td>Lung ADK</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Lung ADK</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Pancreas ADK</td>
<td>+++</td>
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<td>+</td>
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<td>Lung ADK</td>
<td>+++</td>
<td>++</td>
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Table 1: EpCAM-positive MP detection and cytology in pleural effusions from primitive carcinoma
ADK, adenocarcinoma; EpCAM, epithelial cell adhesion molecule; MPs, microparticles; ND, not determined.
Positivity was determined by 1) flow cytometry (FCM) according to the following criteria: negative (−), < 2 MPs/μl; positive (+), 2–50 MPs/μl; (++), 50–100 MPs/μl; and (+++), > 100 MPs/μl. 2) Cytology: negative (−), absence; positive (+), < 5%; (++), 5–50%; and (+++), > 50% of malignant cells, respectively. Grey zones are no
more visible in the table. discordant results between EpCAM + MPs and cytology of malignant cells (+) and 2 more than 50% of malignant cells (+++). Notably, in these last two cases, the expression of EpCAM measured by immunocytochemistry was either negative or weak. Therefore, compared to cytology alone, combining cell detection by cytology and EpCAM+ MPs enumeration by flow cytometry improved the diagnosis of MPE (from 81% to 88%).

**DISCUSSION**

Microvesiculation is a general process which occurs at the membrane of virtually all cell types. Thus, MPs are theoretically present in all body fluids. Accordingly, they have been reported not only in peripheral blood (serum or plasma) but also in other biological fluids such as urine, cerebrospinal fluid, saliva, synovial and vitreous fluids [24]. However, pleural effusions, have been underexplored. Indeed, only the presence of exosomes purified by sequential ultracentrifugations on sucrose or iodixanol density gradient have been described in very small series of patients (3, 9 and 12 patients, respectively [9–11]). In the present study, we report for the first time the presence high amounts of MPs originating from normal and malignant cells in pleural fluids of 85 patients, opening the way for a potential non-invasive biomarker for pleural diseases. Moreover, this study is the first description of tumor-derived MPs expressing the EpCAM antigen in the pleural liquid from lung carcinoma patients. Interestingly, the detection of EpCAM+ MPs by flow cytometry showed a better specificity and sensitivity than ELISA to distinguish between pleural carcinoma and the others malignant pleural effusions. Finally, combining flow cytometric enumeration of EpCAM+ MPs and cytology improved the diagnosis of MPE compared to cytology alone. This study establishes the basis for using EpCAM+ MPs as a promising biomarker that could be add to the armamentarium to distinguish benign and MPE.

EVs offer several benefits over current clinical biomarkers for cancer screening and diagnosis. They shuttle clinically validated biomarkers but they also represent a novel source of proteins and nucleic acids that could be exploited as surrogate biomarkers. Moreover, EVs protect their cargo from the attack of nucleases and proteases, increasing biomarker half-life [25]. In the literature, most of the study demonstrating the biomarker potential of EVs associated with tumor antigen have focused on exosomes [26]. In patients with ovarian cancer, Taylor and colleagues identified a tumor-specific signature of eight miRNAs in EpCAM+ exosomes detectable in patient’s plasma, as a disease-specific signature that discriminate cancer from benign ovarian disease [25]. A retrospective study in stage III and IV melanoma patients showed increased levels of caveolin-1- EVs in plasma with a sensitivity of 69% and specificity
of 96.3% while levels of serum LDH were altered only in 12.5% of patients [27]. Serum prostate-specific antigen (PSA) has been found on plasma- and urine-derived exosomes in prostate cancer [28, 29]. In another report, exosomal survivin was identified as promising surrogate biomarker for early diagnosis of prostate cancers [30]. Plasma levels of survivin-positive-EVs were higher in prostate cancer patients than benign hyperplastic patients and healthy donors, potentially providing an alternative tool to reduce the number of false positives generated by the PSA test. The tumour-specific EGFRvIII was detected in serum EVs from glioblastoma patients which proved to be useful for monitor patient therapy [31, 32].

Figure 2: Tumoral microparticles in pleural effusion.
Representative flow cytometry graphs of podoplanin A. mucin 1 B. and EpCAM C. labeling on MPs from benign B. or cancer C. pleural fluids. The control experiments with appropriate isotype antibodies are displayed above each specific graph. D. The specificity of EpCAM+ microparticles in malignant pleural effusions. Representative experiment of AnnV+/EpCAM+MP labeling by flow cytometry before and after immunomagnetic separation (IMS) using beads coated with the EpCAM antibody. The control IMS was performed with beads coated with an irrelevant antibody. E. The EpCAM antigens are vectorized by MPs. Comparison of the EpCAM antigen determined by ELISA between the pleural fluids, MP pellets and last-wash supernatants (SN) (n = 5).
Taken together, most of these studies supporting the clinical potential of EVs as biomarkers for screening and early diagnosis of cancer have focused on exosomes, which detection is based on time-consuming assays such as western-blot or methods with limited availability such as micro- NMR [32]. In the present study, we identified MPs as valuable markers which have the advantage to be directly accessible by flow cytometry [15], a methodology largely available in diagnostic labs and providing a result in less than one hour. In addition, compared to plasma, the low background noise of the pleural fluid samples is an optimal preanalytic condition for MP measurement. In the present study, we showed that detection of tumoral-MPs by flow cytometry showed a better sensitivity than by ELISA to distinguish between pleural carcinoma and the others MPE. This difference may rely on the pre-analytical steps which differs between flow cytometry and ELISA; the later method includes a high speed centrifugation known to impact on the recovery of MPs whereas flow cytometry is directly performed on the native sample. Although the sensitivity and specificity of a diagnosis by tumoral MPs should be established in larger multicenter cohorts, this study establishes the basis for the detection of tumoral MPs by flow cytometry as potential biomarkers for the classification of pleural effusions.

Cytology is the gold standard method for the diagnosis of pleural effusion but presents limitations that rely on cell scarceness or difficulties to discriminate cancer cells from reactive mesothelial or inflammatory cells [33]. Thus, additional methods have been evaluated to improve the diagnostic accuracy and to avoid invasive diagnostic techniques, such as thoracoscopy [34]. Notably, a combination of cytology and RT-PCR analysis of CEA and Ep-CAM significantly improved the detection sensitivity of tumor cells in serous effusions [35]. In the present study, we showed that combining flow cytometric enumeration of EpCAM+ MPs and cytology improved the diagnosis of MPE compared to cytology alone. Detection of tumoral- MPs by flow cytometry offers also several advantages compared to cytology: 1) tumor-derived MPs are detectable despite a low number of tumoral cells due to the high amounts of MPs produced by tumor cells; 2) tumor-derived MPs are still detectable when the apoptotic parental cells are no longer detectable; therefore, MPs remain detectable in the pleural fluids after the cell degradation which is of particular interest in daily practice for old samples; 3) Detection of tumoral-MPs by flow cytometry is operator-independent and do not necessitate a cytopathological expertise. Beside allowing to mini-invasively discriminate benign and malignant pleural effusion, the detection of EpCAM+ MPs could be useful to monitor patient’s
treatment. Indeed, identification of patient with positive MPs for EpCAM allows to elicit candidate which may benefit from anti-EpCAM biotherapy such as catumaxomab [36, 37].

Because immunohistological detection of EpCAM in pleural fluid is usually difficult in case of low percentage of malignant cells, detection of MPs for EpCAM by flow cytometry may represent an advantageous companion test for personalized medicine targeting EpCAM. This biomarker could also be useful for mini-invasive monitoring of the patients during a specific treatment. In fact, patients with important comorbidities, poor performance status, or advanced age cannot easily undergo thoracoscopy. For the patient, a mini-invasive thoracentesis or the use of indwelling pleural catheter to remove pleural fluid could provide material suitable for a detailed analysis of the microparticles to obtain additional information about the status of the disease.
A limitation of this work is that the detection of tumoral-MPs was restricted to EpCAM+ events. EpCAM antigen is not expressed by all tumors, even not all carcinoma [38] which may explains false negative results for tumoral-MPs detection despite the presence of tumoral cells by cytology. Consistent with this hypothesis, in the two patients negative for EpCAM+ MPs and positive by cytology, the expression of EpCAM measured by immunocytochemistry was either negative or weak despite a large number of malignant cells. So we can assume that detection of malignancy markers on the surface of MPs could be enlarged to the detection of others tumoral antigens such as PSA, EGFR-vIII, CD24 or mesothelin.

This work demonstrating the presence of tumoral MPs expressing EpCAM in pleural fluids also opens new directions about the MPs role in cancer progression. Indeed, in patients with gliomas, it has been reported that EpCAM overexpression correlates significantly with malignancy (WHO grades), proliferation (Ki67), angiogenesis, and prognosis [39]. On the other hand, MPs vectorize nucleic acid molecules such as miRNAs, miRNAs, ncRNAs, DNA, cDNA and retrotransposons originating from parent cells. The recent discovery of EpCAM involvement in cell signaling and breast cancer invasion suggests that its vectorization by MPs could contribute to carcinogenesis [40]. Since tumoral MPs also carry various features (including procoagulant, proteolytic or inflammatory activities) and genetic signatures implicated in malignant responses, it can be assumed that EpCAM+ MPs could behave as relevant players of carcinogenesis in the pleural microenvironment.

PATIENTS AND METHODS

Patients

Eighty-five patients (30 females and 55 males) with pleural effusions were prospectively included in this study. Among them, 71 consecutive patients with histologically-proven primary or metastatic pleural cancer were included. There were 44 patients with carcinoma and 27 patients with others pleural cancers (16 mesotheliomas, 4 melanomas, 3 lymphomas, 2 breast cancers, 1 uterine sarcoma, 1 ovarian cancer). In addition to these patients diagnosed with malignant pleurisy, we enrolled 14 patients with cytologically negative pleural effusions without neoplastic or atypical cells. These patients were not significantly different in gender and age with cancer patients. Informed consent was obtained from the patients or their relatives, and the study was approved by the institutional ethical committee according to local regulation. The cytological diagnosis was performed by an expert cytopathologist, and the findings were reported as “cytologically-positive” in case of significant atypical cells or malignant cells in the pleura and “cytologically-negative” in the other cases [13]. Thoracoscopy was standardized
in accordance to current European practice and used a standardized telescope (R. Wolf Gmbh, Knittlingen, Germany) to obtain multiple parietal pleura biopsies for the histological diagnosis [14].

**Sample processing**

The pleural fluids were centrifuged at 300 g for 10 min and at 1,000 g for 15 min at room temperature with light braking in order to remove cells and avoid artifactual generation of MPs. The collected supernatant was stored at −80°C until use. For ELISA, the MPs were purified by high-speed centrifugation at 70,000 g for 90 min at 4°C as already published [15]. The pelleted MPs were washed twice (70,000 g for 90 min at 4°C) and re-suspended in phosphate-buffered saline (PBS). The supernatant from the last washing was used as the negative control.

**MP enumeration by flow cytometry**

The MPs were enumerated by highly sensitive flow cytometry, as previously described [16]. Briefly, 30 μL of pleural fluid were incubated with the appropriate amount of specific antibodies and 10 μL of AnnV-FITC (fluorescein, Beckman-Coulter, Miami, FL, USA). A fluorescent-matched irrelevant antibody was used as a control for the specific antibodies. Each stained sample was analyzed on a NAVIOS 3-laser instrument (Beckman-Coulter), following a protocol standardized with Megamix-Plus FSC beads (BioCytex, Marseille, France). The platelet-, erythrocyte-, leukocyte- and endothelial-derived MPs were defined as AnnV+/CD41+, AnnV+/CD235a+, AnnV+/CD11b+ or AnnV+/CD31+CD41−, respectively. Positivity for podoplanin (PE conjugated, phycoerythrin; Biolegend, San Diego, USA), EpCAM (PE; clone EBA-1, BD Bioscience, San Jose, USA), and mucin 1 (FITC conjugated; eBioscience, San Diego, USA) was determined by the AnnV+ events. The absolute MP counts (events per μL) were determined using ad hoc counting beads (CytoCount®, Dako, Copenhagen, Denmark).

To verify the specificity of the EpCAM signal using flow cytometry, the EpCAM+ MPs were depleted by immunomagnetic separation using beads (8 × 10⁶ beads/ mL; Dynabeads, Invitrogen, Oslo, Norway) coated with purified EpCAM (clone KS1/4; BD Biosciences). The control experiments were performed in parallel using beads coated with a non-specific antibody (IgG1, 8 × 10⁶ beads/mL; Dynabeads, Invitrogen).

**Cryo-transmission electron microscopy (TEM)**

For the cryo-TEM experiments, 4 μL of pleural liquid was deposited on an electron microscopy (EM) grid coated with a perforated carbon film (Ted Pella, USA). The excess liquid was blotted off with filter paper, and the grid was quickly plunged into liquid ethane in a Leica EM-CPC cryo-chamber. The EM grids were stored in cryo-boxes under liquid nitrogen until use. For the
cryo-TEM observations, the EM grids were mounted in a Gatan 626 cryo-holder and transferred into a Tecnai F20 microscope operated at 200 kV. The images were recorded with a USC1000- SS CCD Gatan camera.

**Measurement of EpCAM-positive MPs using ELISA**

EpCAM was measured by a DuoSet ELISA (enzyme linked immunoSorbent assay) kit (R&D Systems, Minneapolis, MN, USA) in the pleural fluids or purified MPs according to the manufacturer’s instructions. Mouse anti-human EpCAM, at a concentration of 4 μg/mL in phosphate-buffered saline (PBS), was coated on 96- well Costar EIA plates and incubated overnight at room temperature. Then, 100 μL of pleural fluid, purified MPs or corresponding last-wash supernatant were incubated at room temperature for 2 h. Following the washing steps, EpCAM was detected with a biotinylated/peroxidase-streptavidin goat anti-human EpCAM antibody (200 ng/mL). The concentration was determined by measuring the optical density at 450 nm. According to the sensitivity of this test, results > 2 pg/mL were considered positive.

**Statistical analysis**

The statistical analyses were performed with GraphPad Prism software v.5.0 (GraphPad Software, San Diego, CA, USA). The continuous variables are reported as medians and the 25–75th interquartile ranges. A Mann- Whitney test was used for the quantitative variables. The reported p-values are 2-sided, and p < 0.05 was considered significant.

**ACKNOWLEDGMENTS**

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**FUNDINGS**

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**CONFLICTS OF INTEREST**

C. Judicone and
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ARTICLE 2

The diagnosis of neoplastic pleurisies remains difficult because of frequent false negative cytological analysis. Thoracoscopy remains the gold standard to distinguish benign from malignant pleural effusion, but it is invasive and not suitable for all patients. Microparticles (MPs) are promising potential biomarkers and could represent a new approach to identify patients with malignant pleural effusions. We have recently reported the presence of EpCAM-positive-MPs (EpCAM+MPs) in malignant pleurisies that could be routinely used as a complementary tool with cytology for the diagnosis of pleural malignancy.

Here we describe three cases of pleural cancer patients negative for cancer cells but positive for EpCAM+MPs in the pleural fluid. This study confirmed the possibility to apply in clinical practice recent data about EpCAM+MPs, detected in pleural fluid, as a non-invasive pleural biomarker useful to increase sensitivity of cytology. In particular, we suggest in patients with a strong suspicion of pleural cancer to search pleural EpCAM+MPs as a complementary diagnostic tool in case of negative cytological analysis of pleural fluid.
False negative pleural cytology in patient with malignant pleurisies: is pleural EpCAM-positive microparticles a complementary tool for diagnosis?

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INTRODUCTION

Non-invasive biomarkers to differentiate benign from malignant pleural effusion are needed (1, 2). If thoracentesis is usually the first step to achieve cytological analysis in patients with pleural effusion thoracoscopy remains the gold standard to obtain tissue for final diagnosis (3). However, cytology can produce false negative results and thoracoscopy is an invasive approach not suitable for all patients.

Microparticles (MPs) are extracellular vesicles released by all cell types and are considered promising potential biomarkers for diagnosis, prognosis, and disease monitoring. These extracellular vesicles with a size of 0.1-1 micron, originate from blebbing of cell membranes after cell activation or apoptosis. They, generally express the anionic phospholipid phosphatidylserine, and membrane antigens derive from their parental cells (4). Microparticles are also released by tumor cells (5-9). However, very few data have reported the presence of these MPs in pleural fluid (10-12). We have recently reported the presence of EpCAM-positive microparticles in pleural fluid that could represent a new approach to non-invasively identify patients with malignant pleural effusions (13).

Here we describe three cases of cancer patients negative for cancer cells but positive for EpCAM-MPs in the pleural fluid after thoracentesis, illustrating a potential interest of this analysis to improve the diagnosis of patients suffering from malignant pleurisy.
CASE REPORTS

Case 1
A 66-year-old man presented with dyspnea and a pleural effusion on the left side at chest radiograph. His past medical history was significant for head and neck cancer. Physical examination revealed a good performance status (PS 1, ECOG classification), no weight loss, and, at the lower part of the left hemithorax, decreased breath sounds, and dullness to percussion. A CT scan showed a left-sided pleural effusion, and no pleural or parenchymal abnormalities were noted. The patient underwent thoracentesis and the cytological analysis did not detect the presence of cancer cells. Given the high suspicion for relapse of cancer disease, thoracoscopy was performed. It revealed diffuse pleural nodules suggesting metastases and about 2300 ml of hematic pleural fluid were drained (Fig. 1a). We searched the presence of EpCAM positive MPs in pleural fluids using highly sensitive flow cytometry. As illustrated in figure 2a, compared to control, a huge number of MPs positive for EpCAM was detectable in the pleural fluid of this patient (Fig. 2, case1). Although the cytology was negative, the histological report of pleural biopsy was consistent with the diagnosis of metastasis from head and neck cancer.

Case 2
A 67-year-old patient visited the outpatient clinic for a right pleural effusion documented at chest radiography. He had never smoked. Physical examination revealed a good performance status (PS 0, ECOG classification), and it was unremarkable. The thoracentesis performed to improve its symptoms showed after biological analysis an exudate without malignant cells. A chest CT scan confirmed right pleurisy without pulmonary or pleural lesions. The patient underwent thoracoscopy for diagnostic and medical purposes. Pleural biopsies were taken and a pleurodesis using dedicated talc was carried out due to the strong macroscopic suspicion of malignant disease (Fig. 1b). Compared to control, EpCAM positive MPs were also detected in pleural liquid by cytometry (Fig. 2, case2). The histological diagnosis of pleural biopsy revealed metastasis from adenocarcinoma of probable prostatic origin. A total body CT scan showed a prostate cancer with bone metastasis.

Case 3
A 37-year-old woman with a significant smoking history presented with dyspnea, fatigue, and weight loss. The physical examination revealed dullness to percussion with decreased breath sounds at the lower part of the left hemithorax.
A left-sided pleural effusion was identified on chest X-ray and confirmed by the chest CT without lung or mediastinal abnormalities. Thoracentesis yielded pleural fluid consistent with an inflammatory pleurisy without malignant cells.

Compared to control, EpCAM positive MPs were detected in pleural liquid by cytometry (Fig. 2, case 3), at a level comparable to patient 2.

By thoracoscopy 500 ml of citrine pleural liquid were removed and pleural samples were taken (Fig. 1c).

Pleural biopsies during the procedure using an optical biopsy forceps confirmed the diagnosis of pleural metastasis from breast cancer. Mammography identifies a nodular lesion at the level of the left-upper-outer lobe strongly suggestive of breast cancer.

**DISCUSSION**

Cytological analysis has variable diagnostic yields depending on multiple factors and situations. However, according to the recommendations, thoracentesis for sampling the fluid still remains the first step leading to cytological analysis for the diagnosis of pleural cancer. Indeed, in pleurisy, it is largely used to distinguish benign from malignant pleural effusion. Nevertheless, this technique presents some limitations. In fact, the cell poorness or the difficulties to distinguish tumor cells from reactive or inflammatory mesothelial cells can cause false negative results (14).

At contrary, thoracoscopy allowing pleural biopsies usually achieves the diagnosis between benign and malignant effusion. For this reason it is the gold standard technique in case of pleural abnormalities (nodules, thickenings...) and pleural effusion after imaging procedures. However, thoracoscopy has some limitations and cannot be applied for all patient. In fact, advanced age, poor performance status, and comorbidities can limit this invasive procedure.

The presence of specific microparticles in the pleural fluid could be considered as promising non-invasive biomarkers beside the cytological analysis of the fluid and can increase the diagnostic yield.

Microparticles can easily be detected from different body fluids, including peripheral blood, urine, cerebrospinal fluid, saliva, or synovial and vitreous fluids (15).

Moreover, MPs contain many antigens also present in the cell of origin (16-17). For this reason, they could considered useful as biomarkers for the screening and the diagnosis of a cancer at an early stage (18-23).

In a recent study, we documented the presence of high amounts of normal cells-derived and tumor-derived MPs in pleural fluids (24). In this study, we found EpCAM+ MPs deriving from cancer cells, which identified patients suffering from carcinoma although the pleural cells were no longer detectable. Therefore, EpCAM+MPs could be considered as promising biomarker complementary to cytology to distinguish benign and malignant pleurisy (Fig 2).
For the first time we reported here three clinical cases of cancer patients negative for cancer cells at pleural cytology but positive for EpCAM-MPs, illustrating the potential of MPs detection to improve the diagnosis of patients suffering from malignant pleurisy (Fig. 3).

These clinical cases show the possibility to apply a non-invasive method, based on the detection of pleural EpCAM-positive MPs, which is complementary to pleural cytological analysis.

Therefore, even if the cytological analysis is negative, the detection of EpCAM-positive MPs could be added to cytology to better discriminate benign from malignant pleural effusions. The positivity of MPs in patient with negative cytology could be explained by the diffusion properties of the vesicles in the pleural fluid. In addition, the fact that MPs, deriving from apoptotic cancer cells, remains present even if parental cells are no longer detectable because of the process of apoptosis. Moreover, in contrast to cytology it is important to remember that flow cytometry is an operator-independent technique allowing to count MPs.

The MPs also contain protein nucleic acids and therefore carry a genetic signature and could be implicated in carcinogenesis. In particular, the MPs have a role in the progression of the disease, in cellular function and in genetic regulation. Further molecular biology techniques applied on MPs of neoplastic pleurisies could be interesting to study their genetic signature and their carcinogenic potential. Therefore, this present report opens new perspectives about the utility of MPs from pleural fluid. Moreover, it offers the prospect of designing new therapeutic approaches.

In particular, since anti-EpCAM target therapy are available (25), it could be of interest to identify patients with EpCAM-positive MPs to define more personalized therapeutic approaches (26).

**CONCLUSIONS**

We have recently reported that EpCAM+MPs detected in the pleural fluid are correlated with the presence of neoplasia and, in particular, with adenocarcinoma type. These reported cases illustrate how this marker can complete the usual cytological analysis of the pleural fluid.

Consequently, pleural EpCAM+MPs combined with pleural cytological analysis could be relevant for poor performance status patient for whom more invasive procedure is not available in case of suspicion of pleural cancer.

Moreover, since cancerous MPs have also several activities and genetic signatures related to malignant responses, EpCAM+ MPs could be involved in carcinogenesis of the pleural microenvironment. Finally, EpCAM+MP detectable in pleural fluid could act as a target for specific treatment as already reported in others cancers (27, 28).
Figure 1: Thoracoscopic features of the three clinical cases.

(lung: ●, parietal pleura: ♦, diaphragm: □)

a: Case 1 - Metastatic pleural cancer with huge nodules and neoplastic lymphangitis.
b: Case 2 - Pleural metastasis of prostatic adenocarcinoma.
c: Case 3 - Costo-diaphragmatic gutter in patient with breast cancer, multiple nodules are disseminated on the parietal pleural (♦) and on the diaphragm(□).

Figure 2: EpCaM-positive microparticles in pleural effusion.
Representative flow cytometry graphs of EpCAM labeling on MPs from pleural fluid. The control experiments with appropriate isotype antibodies are displayed above.
REFERENCES

2.3 DISCUSSION

A pleural effusion is an abnormal collection of fluid in the pleural space resulting from excess fluid production or decreased absorption or both. It is the most common manifestation of pleural disease, with aetiologies ranging from cardiopulmonary disorders to symptomatic inflammatory or malignant diseases requiring urgent evaluation and treatment.

Determining the etiology of a pleural effusion is facilitated by analysis of the pleural fluid. A systematic approach to analysis of the fluid in conjunction with the clinical presentation should allow the clinician to diagnose the cause of an effusion in about 75 percent of patients at the first clinical evaluation.

Pleural fluid sampling and cytological examination should be undertaken in all patients with a suspected MPE, and will provide a diagnosis in up to 60% of patients. Thoracentesis is a simple bedside procedure with imaging guidance that permits fluid to be rapidly sampled, visualized, examined microscopically, and quantified for chemical and cellular content.

If cytology does not yield the diagnosis, then histological diagnosis should be obtained via pleural biopsy. Percutaneous biopsies US-guided or CT-guided and thoracoscopy are carried out to obtain tissue for histological analysis.

Malignant pleurisy is very common in patients with cancer reflecting the dissemination of malignancy and the advance disease. Moreover, MPE represent a significant health burden and are an important cause of cancer-related mortality and morbidity.

However, the differential diagnosis of pleural effusion remains difficult and the most important aim is to distinguish between malignant and benign pleural effusion. Currently, the differential diagnosis is based on mini-invasive approaches such as thoracoscopy with a high diagnostic yield. The thoracentesis is the first step and it’s useful, but it can present several false negative for cancer cells. Therefore, the thoracoscopy remains the gold standard, even if it cannot be applicable for all patients, because of their comorbidities, their performance status, and their age. Anyway, non-invasive biomarkers that can discriminate between benign and malign pleurisy are needed in particular during the patient’s follow-up.

Various biomarkers have been proposed as noninvasive tests to help distinguish between benign and malignant pleural disease, and to give information on prognosis or treatment outcomes. Multiple serum and pleural biomarkers such as mesothelin, osteopontin and fibulin-3 have been investigated in MPE. In general, these biomarkers have demonstrated poor specificity and sensitivity and results have not been validated in subsequent studies. Even if several biomarkers were tested their sensitivity and specificity seem to...
be not enough for the differential diagnosis between benign and malignant pleural effusion. Moreover, their assay is not widely available outside the research setting and there are limited data in this area.

Among possible non-invasive markers, MPs could be represent interesting future perspective. They are extracellular vesicles which result from the blebbing of cell membrane in response to activation or apoptosis, and are released by all eukaryotic cells, including cancer cells\textsuperscript{344,408}. They are generally characterized by a size comprised between 0.1 and 1 micron, the expression of the anionic phospholipid phosphatidylinerine (PS) membrane antigens representative of their parental cells so that their origin can be determined by using specific antibodies and annexin V\textsuperscript{344}. These characteristics distinguish MP from exosomes which are smaller in size. The presence of MP and exosomes have been reported in human body fluid including plasma and other liquids such ascites fluid\textsuperscript{300,319,868}. However, there are only few data available about extracellular vesicles in pleural liquid\textsuperscript{325,869}.

Pleural effusions are very common in both benign and malignant diseases and MPE characterizes several cancer metastasizing in the pleural space. However non-invasive biomarkers are needed to achieve the differential diagnosis avowing interventional procedures. Given that the MPs are considered innovative biomarkers and there are many data of their detection in the blood, we have assumed their presence also in the pleural fluid. In particular, because tumour cells produce high amount of MPs\textsuperscript{870}, we hypothesized that TMPs, detected in pleurisies, could help to identify in a non-invasive way patient with malignant pleural effusions.

First of all, we performed pre-analytic steps, consisted of handling and storage of pleural samples, because to our knowledge there were no data in literature on methods to detect MPs from pleural liquid. Therefore, the definition of protocols for manipulation and storage of pleural samples was needed because it is important to obtain good samples to detect interesting events (MPs), avoiding background noise. Anyway, in literature there are very few data about these procedures. Thus, we tested different methods in order to obtain the optimized protocol (Article 1 and Annexe 1).
Because few data were available about extracellular vesicles in pleural liquid, after the first step, we checked existence of pleural MPs. Therefore, we looked for their presence using flow cytometry. In particular, we looked for extracellular vesicles with a size included within an analysis window defined by 0.3 to 0.9 µm beads and positive for annexin-V+ labelling, detectable in pleural fluid, both from neoplastic and non-neoplastic aetiology. These features were defined as compatible with MPs definition (Article 1 and Annexe 1).

In order to characterize the cellular origin of these MPs from pleural effusion, we first performed complementary immunophenotyping with antibodies and we found MPs derived from erythrocytes (EryMP), platelets (PMP), leukocytes (LMP) and endothelial cells (EMP). We detected extracellular vesicles with light scatter properties compatible with those of MPs and positive for Ann-V+ derived from pleural fluids of neoplastic and non-neoplastic etiologies. We note that there were no significant differences in the total MPs count between benign and MPE, using Ann-V labeling. This is probably related to the fact that the main processes of formation of MPs, cell activation and apoptosis, are common both to inflammatory pleurisy and pleural effusion consequent to cancer. In addition, the level of MPs from hematopoietic and vascular origin which does not differ between benign and MPE. These results agree with the notions that inflammation and vascular activation are common features of pleurisies regardless of origin.

After this, we analysed whether TMPs were present in MPE. We tested the composition in these MPs subpopulations and we checked whether they were significantly different between malignant and benign pleural effusions. Thus, we defined the origin of MPs, and we showed for the first time the presence of TMPs in this biological compartment.

Indeed, we hypothesized that tumour-derived MPs could be present in pleural liquid and could help to identify in a non-invasive way patient with malignant pleural effusions. Therefore, the presence of selected tumour associated markers on pleura was tested. For this reason, we used specific antibody labelling for neoplastic markers. Indeed, we analyzed the most common immunohistochemical markers used in the differential diagnosis between epithelioid pleural mesothelioma and lung adenocarcinoma. Among them, we
chose surface markers which are present at the cell membrane and, thus, potentially present at the MP surface: podoplanin, mucin 1 and EpCAM. Podoplanin+MPs and mucin 1+MPs were found in pleural effusions of both cancer and benign origin. This is consistent with the expression pattern of podoplanin found to be upregulated in mesothelioma and other human cancers, but also in mesothelial cells and other normal tissues. Similarly, mucin 1 can be expressed in tumoral and normal tissues including lung, mammary gland, uterus, esophagus, stomach, duodenum, pancreas, prostate, and hematopoietic cells. Therefore, both podoplanin and mucin 1+ failed to assign the malignant etiology of pleural fluid. By contrast, MPs expressing EpCAM+ were detected in malignant pleural effusions only. Therefore, in pleural liquid there is a large amount of MPs derived from normal cells, but also pleurisies can contain TMPs. In particular, we demonstrated the presence of tumor-derived microparticles expressing EpCAM in the pleural fluids from adenocarcinoma patients. In particular, we analysed the EpCAM antigen as marker of adenocarcinoma and this was the first work to directly identify tumoral microparticles in pleural liquids using EpCAM. We hypothesized that detection of EpCAM+ microparticles could lead to a new diagnostic and monitoring approach. This study established the basis for a potential biomarker for diagnosing and monitoring malignant pleurisy. Moreover, we analysed whether there are specific antigens on MPs surface useful to better define malignant pleurisies, and to identify a specific subgroup of patients. In particular, we analysed the EpCAM antigen as marker of adenocarcinoma. In addition, we think that this biomarker could be useful also for non-invasive monitoring of the response to treatment. Therefore, we hypothesized that the detection of specific MPs may be considered as a complementary tool with the cytology to better discriminate benign from tumoral origin of pleural effusion. As it is well known, malignant pleural effusion is very common in neoplastic patients. Non-invasive biomarkers to differentiate benign from malignant pleural effusion are needed. Indeed, nowadays, thoracentesis is the first step useful to achieve cytological sample, but
cytology can produce false negative results. Moreover, thoracoscopy is an invasive approach not available for every patient, anyway it still considered the gold standard to obtain tissue\(^{190}\). In this context, MPs are extracellular vesicles released by all cell types that are promising potential biomarkers for diagnosis, prognosis, and disease monitoring. This could represent a new approach to non-invasively identify patients with malignant pleural effusions.

In addition, as it is well known, tumoral MPs (TMPs) are documented in several studies, even if very few data have reported the presence of these MPs in pleural fluid\(^{300, 301, 319, 869, 893}\). Moreover, in a recent publication, we showed the possibility to identify adenocarcinoma patients, detecting the EpCAM positivity of MPs from pleural liquid\(^{894}\).

For this reason, in another work, we analysed three cases of cancer patients negative for cancer cells but positive for EpCAM-MPs in the pleural fluid after thoracentesis (\textit{Article 2}). This finding illustrates the interest of this analysis to improve the diagnosis of patients suffering from malignant pleurisy\(^{344}\).

Indeed, in all the clinical cases we reported, the cytological analysis obtained by thoracentesis was negative for neoplastic cells. For all these patients, flow cytometry and ELISA test were carried out and in all reported cases EpCAM+MPs were found in their pleural liquid. Therefore, because of the suspicious of relapse of their anamnestic cancer, these patients underwent thoracoscopy and the pleural biopsy achieved the diagnosis of cancer.

This study confirmed the possibility of considering EpCAM-positive MPs as a non-invasive biomarker useful to increase sensitivity of cytology. Indeed, we suggest that, in case of cytological analysis negative for cancer cells, if a malignant pleural effusion is suspected, the detection of EpCAM-positive MPs in pleural fluid could be useful to improve diagnosis.

In addition, this analysis could be interesting for mini-invasive monitoring of patient’s treatment, for obtaining additional information about the status of the disease.

We showed that MPs could be complementary to routine analysis and they could be useful to improve diagnosis. We identify patients suffering from cancer positive for EpCAM-MPs detected in pleural fluid, but with negative cytology for malignant cells. (\textit{Article 2}).

EpCAM+ MPs could be involved in the role in carcinogenesis of the pleural microenvironment. Therefore, promising therapeutic approaches could be considered as target therapy against EpCAM+MP detectable in pleural fluid. This interesting biomarker would help the oncologist to identify the subpopulation of patients that may benefit from new therapeutic approach.

Moreover, it could be interesting to test other labelling of different tumoral biomarkers (PSA, CEA, CA15.3, CA19.9, NSE, Mesothelin…). These experiments may allow to define MPs positive for these markers, eventually involved in the pathogenesis of these tumors. This could
allow to expand the method that we have applied to adenocarcinoma by EpCAM+MPs to several cancers histologies by other TMPs positive for different tumoral biomarkers.

Our studies opens a new horizon to future perspectives. For example, it might be interesting to investigate eventual specific activities of MPs, probably involved in tumor pathogenesis. Additionally, given that MPs are characterized by a complex cargo, it could be useful to analyse their "genetic signature". Furthermore, testing different neoplastic markers on MPs, it could be possible to define their diagnostic, prognostic and predictive value, and to eventually design new therapeutic approaches against tumoral MPs.

Activities of MPs
Several data report that MPs had interesting activities, probably related to the pathogenesis of disease, even if additional studies are needed to confirm such statement. Nowadays, different reports suggest a pathophysiological implication of tumoral MPs in thrombosis of cancer patients, but the causal link has not yet been fully established. The fibrinolysis was also described on MPs that represent catalytic surfaces capable of converting plasminogen to plasmin. For this reason, it is interesting to evaluate whether MPs in pleural fluid have thrombolytic activity or ability to plasmin generation.

**MPs from pleural liquid: detection of procoagulant and profibrinolytic activities**
We hypothesized that also MPs from pleural effusion have some activities that could be involved in the pathogenesis of thrombosis, very common in cancer patients, and fibrinolysis, probably related with the formation of metastasis. For this reason, we investigated, for the first time, whether pleural MPs are able to form plasmin and to activate the tissue factor.

In this preliminary study, we prospectively enrolled 85 patients with pleural effusions; out of them 30 were females and 55 males. There were 71 consecutive patients suffering from primary or metastatic pleural cancer: 44 patients had carcinoma and 27 patients had others pleural cancers (16 mesotheliomas, 4 melanomas, 3 lymphomas, 2 breast cancers, 1 uterine sarcoma, 1 ovarian cancer). Fourteen patients with benign pleurisy were enrolled as controls. Patients with benign or malignant pleural effusion did not significantly differ in gender and age. (Figure 43).
We detected EpCAM+MPs, using a specific labelling by flow-cytometry on pleural fluid and ELISA test on MPs pellet. The specificity of this analysis was confirmed using immunomagnetic depletion with beads coated with a EpCAM antibody (Figure 44).

Figure 44: EpCAM+ MPs in pleural liquid
The cancer patients develop very frequently serious thrombotic events and, up to today, there are not effective biological markers useful to identify patient that can benefit from anticoagulant treatment, in order to prevent these complications. The TMPs have been described as bearing a procoagulant activity dependent from Tissue Factor (TF). Some authors show that injection of FT+MPs derived from cancer cells in mice causes the disseminated intravascular coagulation syndrome in vivo. Other studies show that injection of TMPs derived from human and murine pancreatic tumor cell lines induces a reduction of time needed for venous and arterial occlusion by a mechanism dependent on P-selectin.

Although a significant number of data and published clinical studies are in favour of a pathophysiological involvement of MPs in these tumor thrombosis-related cancers, a causal link has not yet been fully established in clinical practice. The identification of MPs from pleural fluid capable of producing TF may be useful to better define the pathogenesis of these procoagulant alterations. In addition, MP-TF could be considered as a non-invasive biological marker, easily accessible and reproducible over time. The cancer patients develop very frequently serious thrombotic events and, till today, there are not effective biological markers useful to identify patient that can benefit from anticoagulant treatment, in order to prevent these complications. The identification of MPs from pleural fluid capable of producing TF may be useful to better define the pathogenesis of these procoagulant alterations. In addition, MP-TF could be considered as a non-invasive biological marker, easily accessible and reproducible over time.

Therefore, we tested the ability of MPs to activate TF with a sensitive factor Xa activity assay with or without inhibitory TF antibody. As shown in figure number 45, the TF activity of MPs (MP-TF) was significantly increased in malignant pleural effusion, compared to benign pleurisies.

**Figure 45: TF activation by MPs**
MP-TF activity: sensitive factor Xa activity assay w/wo TF inhibitory Abs.
*(MP-TF: activation of TF by MPs; B: Benign pleural effusion; C: Cancerous pleural effusion.)*
The proof of concept of the role of MPs in the proteolysis was initially demonstrated from MPs derived from malignant tumor cells, which have an important proteolytic potential, characterized by the production of TMPs proteolytic factors, necessary for their release and tissue invasion\textsuperscript{656}. In particular, TMPs can vectorise uPA and uPAR receptor, proMMP-2, proMMP-9 and their active forms, as well as complexes between MMP's and TIMP-1 inhibitor. Moreover, the process of plasmin generation can be regulated by PAI-1, AP-1 and α2antiplasmine. The presence of proteolytic enzymes on the membrane of TMPs was identified in vitro and in vivo, for example in cancer of ovary and prostate\textsuperscript{576, 657}. Therefore, the ability to generate plasmin could be correlated with the presence of metastases and the ability of tumor cells to invade the plasma membrane and move away. Indeed, all cancer patients enrolled in this study had advanced disease and had pleural metastasis.

Thus, we analysed the capacity of MPs to generate plasmin with a IMS based assay with a specific substrate for plasmin. As shown in figure number 46, the generation of plasmin of MPs (MP-PGC) also in this case was significantly increased in malignant pleural effusion, compared to benign pleurisies.

\textbf{Figure 46: Plasmin generation by MPs}
MP-Plasmin generation capacity: IMS-based assay with specific plasmin substrate. 
\textit{(MP-PGC: generation of plasmin by MPs; B: Benign pleural effusion; C: Cancerous pleural effusion.)}

We also characterized the original cell of MPs present in pleural fluid, and we found MPs from platelets (Plt), from erythrocyte (Ery), from endothelial cells (Endo), from leucocytes (Leu), and from tumoral cells positive for EpCAM labelling. MPs activities seem to be related with the different identified subsets of MPs. Interestingly, when we correlated MPs activities to the different identified MP subsets, we found the best correlation coefficient with EpCAM MPs both for the TF activity and the plasmin generation capacity. This suggests that this activity mainly originate from the tumoral MPs in pleural fluid.
In a recent study, we identify MPs from pleural liquid and we characterized their original cell. In particular, we recently defined for the first time MPs from tumoral cells positive for EpCAM labelling. Instead, these preliminary data showed for the first time that MPs from pleural fluid are able to form plasmin and to activate the tissue factor. These findings open new perspectives that can be useful to better understand the pathogenesis of the microparticles and in particular of those released in the pleural fluid. These features could offer the possibility of an early diagnosis of thrombotic events, of the identification of patients who can benefit from prophylactic anticoagulant treatment, and of the monitoring of the disease over time. More studies are needed to confirm these results and to clarify these processes and the role of the functional activity of the MPs of pleural fluid in tumor pathogenesis.

**MPs from different body fluids: correlations and discrepancies**

An interesting question concerns the comparison of MPs between different body fluids from the same patient. In particular, it could be interesting to analyse the correlations or discrepancies between different biological compartments, like pleural fluid, bronchoalveolar lavage, and peripheral blood.

In this project, we had already stored all biologic fluid and thus, it will be possible to analysed the MPs detected in these compartments, using the manipulations verified by laboratory protocols in our feasibility study.
In a next project, we will compare MPs present in pleural fluid with eventual circulating MPs in blood and in bronchoalveolar lavage, at the same time of thoracoscopy/thoracentesis. These findings can define whether MPs detected in these different substrates have common characteristics or they provide different information.

"Genetic signature" of MPs from pleural effusion

MPs contain proteins but also nucleic acid including mRNAs, miRNAs, ncRNAs, genomic DNA, cDNAs, and retrotransposons. All these genetic materials are implicated in disease progression, in cell function and gene regulation and they are present in several neoplastic MPs. For this reason, we would assess whether the MPs obtained by pleural effusion have a "genetic signature", to better define their genetic characteristics. We will analyse microRNA which, as it is well known, can be extrapolated by microparticles and we will perform tests of molecular biology.

Diagnostic, prognostic and predictive value of MPs

Whether it is possible to define the "gene signature" of the biological material extracted by MPs, it would be interesting to evaluate the information of transported genes, especially those carried by neoplastic microparticles. Thus, we could evaluate possible diagnostic, prognostic and predictive value of isolated MPs from pleural fluid. MP can be also considered as cancer biomarkers: indeed, they are detected in the circulation of patients with breast, lung, prostate, colorectal, gastric, glioblastoma, ovarian, pancreatic, bladder cancer. Therefore, MPs can be considerate as diagnostic, prognostic, and predictive factors and they could be very useful in clinical practice, also because their collection is often possible through minimally invasive medical techniques. This project could open the way to evaluate diagnostic, prognostic and predictive value of MPs, and to define the origin of metastatic pleural disease using specific tumoral antigen MPs against the primitive cancer (ErB1, PSA, CA19.9, CA15.3, CA125…).
Neoplastic markers on MPs
In the feasibility study, in order to isolate neoplastic microparticles, we started from a labelling of tumor receptors that are more accessible with known laboratory techniques. For this reason, we chose surface markers.
Anyway, it could be very interesting to perform other labelling, which can highlight tumor markers that may be present within the microparticles in order to better characterize these MPs. The analysis of other markers may also be useful for capturing other microparticles that are negative at surface marking, but may be positive for other markers present in the cell and not yet tested (Calretinin, Keratin 5, WT1, CEA…).

Therapeutic approach against tumoral MPs
The most interesting question, in our opinion, is to evaluate the usefulness in clinical practice of the results that we obtained with our research.
Since the presence of neoplastic microparticles in pleural fluid was proven and their possible activity in tumor pathogenesis was documented, we are interested in the design of therapy protocols against target receptors present on the microparticles themselves.
A first approach could be for example to test directly in pleural fluid the target drugs already used in current clinical practice systemically. With this technique, we could try to limit the possible tumor dissemination and locally arrest the neoplasia.
It is important to remember that nowadays anti-EpCAM biotherapies are available, for example catumaxomab.
Therefore, the identification of patients with EpCAM-positive MPs could be useful to define more personalized therapeutic approaches.

In conclusion, my thesis demonstrated for the first time the presence of a large amount of MPs in pleural effusions, originating from a large panel of normal and malignant cells.
In addition, we characterized their cell of origin and this was the first work that directly identified tumour derived MPs in pleural liquid from different aetiologies.
Although the diagnosis sensitivity and specificity of tumoral-MPs need to be establish in larger multicentric cohorts, this study would lay the basis of tumoral antigen MPs as potential biomarkers for classified pleural effusion. Indeed, in our cohort, the detection of EpCAM-positive microparticles were related with adenocarcinoma histology and they could be useful
as non-invasive biomarker. On other side, this study would open the way to define the origin of metastatic pleural disease using specific tumoral antigen MPs against the primitive cancer. TMPs, an in particular, EpCAM+MPs were also shown to be useful as a tool to complement cytology for better diagnoses of malignant pleurisies, also in case of negative cytology for malignant cells.

Moreover, because MPs have been described not only as marker of cellular activation but also as vector of various activities, it is now relevant to study their impact on pleural microenvironment, and cancer pathogenesis.

In addition, these specific MPs can be useful as non-invasive monitor of the response to treatment.

Finally, EpCAM+MPs could be considered as a marker for patients’ subpopulation that may benefit from personalized therapeutic approach.
ANNEXES

ANNEXE 1

European Respiratory Society 2015 International Congress

Abstract Number: 851089 selected as Oral Presentation
Abstract Group: 11.2. Pleural and Mediastinal Malignancies
Keywords: Biomarkers; Lung cancer / Oncology; Pleura

Detection of EpCAM-positive microparticles in pleural fluid:
a new approach for the diagnosis of the tumoral origin of pleural effusions

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BACKGROUND
Pleural biomarkers are needed for the diagnosis of malignant pleural effusion at an early stage before true carcinomatosis. Tumor cells produce microparticles, and, therefore, we hypothesized that tumor-derived microparticles could be present in the pleural liquid and could help to identify patients with malignant pleural effusions by a non-invasive method.

METHODS
Fifty patients with benign (n=11) or malignant (n=39) pleural effusions were included in this study. Among them, 39 consecutive patients with histologically shown primary or metastatic pleural cancer were included. After the optimization of a sample processing protocols, MPs were enumerated by flow cytometry and the Cryo-Transmission Electron Microscopy was performed. Measurement of EpCAM-positive MPs was analyzed by ELISA.

RESULTS
Using highly sensitive flow cytometry and cryo-electron microscopy, this study showed the presence of microparticles in pleural effusions. We demonstrated the presence of tumor-derived microparticles expressing EpCAM (epithelial-cell-adhesion-molecule) in the pleural fluids from adenocarcinoma patients. In our cohort, the detection of EpCAM-positive microparticles was shown to be useful as a tool to complement cytology for better diagnoses of malignant pleurisies.

CONCLUSIONS
To our knowledge, this is the first work to directly identify tumoral microparticles in pleural liquids using EpCAM. The identification of EpCAM+ microparticles could lead to a new diagnostic and monitoring approach. This study establishes the basis for a potential biomarker for diagnosing and monitoring malignant pleurisy.
ANNEXE 2

Tissue factor positive microparticles remain undetectable by flow cytometry in pleural fluids from cancer patients despite high level of procoagulant activity

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Keywords: Tissue factor, Microparticles, Procoagulant activity, Biomarkers

Abstract, ISTH SSC May 2016

Background
The feasibility of detecting tissue factor (TF) expressing microparticles (MPs) by flow cytometry (FCM) is a matter of debate since this approach is prone to artifacts and discrepancies between MP-associated TF-specific procoagulant activity (MP-TF PCA) and FCM-based data have been described. Pleural fluids from cancer patients can harbour EpCAM+ MPs and this has been proposed as an early diagnostic marker for malignant effusions (1). With tumor-derived MPs in these fluids, both a high MP-TF PCA and TF+ MPs (MP-TF) may be expected.

Aims
Both approaches were applied to n=12 samples to compare data.

Methods
Malignant pleural fluid samples were collected as described (1). MP-TF PCA was operated on total MPs extracted from frozen aliquots of pleural fluids using a FXa generation assay modified from Lee, JTH 2011 with sensitivity limit below 10 fM TF (2). FCM detection of MP-TF was standardized on a 3-laser Gallios cytometer with cutoff set at 0.3 µm-eq in FSC (3). Specific care actions (4) included i) multi-color discrimination of MP subsets, ii) addition of hirudin to avoid micro-coagulation, iii) reagents pre-cleaning, iv) negative controls with detergent-based MP lysis.

Results
On 12 fluids with detailed immuno-phenotype, MP-TF remained un-detected despite i) high levels of MP-TF PCA, most often >100 fold higher than normal values in plasma (>1.5 pM TF vs ~15 fM), ii) presence of EpCAM+, likely tumor-derived, MPs.

Conclusions
Bio-assays are more appropriate than immuno-assays to detect MP-TF in pleural fluids. Immunological detection of TF on MPs remains a challenge even though major care is applied in FCM protocols and explosive levels of MP-TF PCA are measured. Whether MP-TF PCA may complement the immunological detection of EpCAM+ MPs for mini-invasive identification of patients with malignant pleural effusions remains to be established.

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Tissue factor positive microparticles remain undetectable by flow cytometry in pleural fluids from cancer patients despite high level of procoagulant activity.

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HEM16

**Background:** The feasibility of detecting tissue factor (TF) expressing microparticles (MP-TF) by flow cytometry (FCM) is a matter of debate since this approach is prone to artifacts and discrepancies between MP-accelerated TF-specific procoagulant activity (MP-TF PCA) and FCM-based data have been described. Pleural fluids from cancer patients can harbour Endo-particles and MP-TF PCA and this has been proposed as an early diagnostic marker for malignant effusions. With tumor-derived MP-TF exceeds MP-TF PCA and MP-TF counts may be expected to be elevated.

**Aim:** Compare FCM-based MP-TF detection with MP-TF PCA to grade the sensitivity of both approaches.

**Clinical samples:**
- Plasma
- Tumor

**Methods:** Multivariate pleural fluid samples collected in described (1) and centrifuged at 3000 x g and 3000 g to remove cells and debris. MP-TF PCA operated on total pleural fluids extracted from a centrifugation of fractionated fluids. Total MP-TF PCA was measured by a magnetic bead assay (MBA) (2) and FCM detection of TF was standardized on a 3-laser FACSCalibur flow cytometer with a 488-nm argon laser in FSC 3 and 4 lasers. Specific features (4) included (1) multi-color differentiation of MP subsets, (2) addition of heparin to avoid microaggregation (5), (3) reagents pre-clearing (20 Qv: 5 min). In negative and positive staining MB controls, total MP negative controls using detergent-based MP lysates (6).

**Conclusion:** FCM detection of TF expressing microparticles positive in the extracellular compartment may offer new perspectives in the early diagnosis of malignant effusions and in the follow-up of patients with pleural metastases.
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References

For a comprehensive list of references, please refer to the cited sources in the text. The references include studies on thrombin generation (TGT), activated factor X, and circulating microparticles in various disease states and conditions.


Additional references are available in the provided text, covering topics such as microparticle quantification, flow cytometry-based analysis, and regulatory control of complement on blood platelets.


