Lymph flow pattern in pleural diaphragmatic lymphatics during intrinsic and extrinsic isotonic contraction

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Moriondo A, Solari E, Marcozzi C, Negrini D. Lymph flow pattern in pleural diaphragmatic lymphatics during intrinsic and extrinsic isotonic contraction. Am J Physiol Heart Circ Physiol 310: H60–H70, 2016. First published October 29, 2015; doi:10.1152/ajpheart.00640.2015.—Peripheral rat diaphragmatic lymphatic vessels, endowed with intrinsic spontaneous contractility, were in vivo filled with fluorescent dextran and microspheres and subsequently studied ex vivo in excised diaphragmatic samples. Changes in diameter and lymph velocity were detected, in a vessel segment, during spontaneous lymphatic smooth muscle contraction and upon activation, through electrical whole-field stimulation, of diaphragmatic skeletal muscle fibers. During intrinsic contraction lymph flowed both forward and backward, with a net forward propulsion of 14.1 ± 2.9 μm at an average net forward speed of 18.0 ± 3.6 μm/s. Each skeletal muscle contraction sustained a net forward-lymph displacement of 441.9 ± 159.2 μm at an average velocity of 339.9 ± 122.7 μm/s, values significantly higher than those documented during spontaneous contraction. The flow velocity profile was parabolic during both spontaneous and skeletal muscle contraction, and the shear stress calculated at the vessel wall at the highest instantaneous velocity never exceeded 0.25 dyne/cm². Therefore, we propose that the synchronous contraction of diaphragmatic skeletal muscle fibers recruited at every inspiratory act dramatically enhances diaphragmatic lymph propulsion, whereas the spontaneous lymphatic contractility might, at least in the diaphragm, be essential in organizing the pattern of flow redistribution within the diaphragmatic lymphatic circuit. Moreover, the very low shear stress values observed in diaphragmatic lymphatics suggest that, in contrast with other contractile lymphatic networks, a likely interplay between intrinsic and extrinsic mechanisms be based on a mechanical and/or electrical connection rather than on nitric oxide release.

lymph propulsion; tissue stress; spontaneous lymphatic contractility

NEW AND NOTEWORTHY

In diaphragmatic lymphatics, flow velocity and lymph flow were more than two order of magnitude greater during contraction of diaphragmatic skeletal muscle than during spontaneous contraction of lymphatic smooth muscles, suggesting a marginal role of the latter in setting lymph flow in rhythmically moving, thoracic tissues.

THE PLEURAL DIAPHRAGMIC lymphatic system is composed of linear lymphatic vessels preferentially located in the tendineous and the medial muscular portion of the diaphragm, and of a more complex net of loop-like structures interconnected by short linear tracts and preferentially located at the most peripheral diaphragmatic rim (18). Given the strategic role played by pleural lymphatics in setting the correct pleural fluid volume and subatmospheric pressure required to maintain the normal lung chest wall coupling (29), much effort has been spent in the study of the inner regulatory mechanisms of lymph drainage and propulsion in this particular lymphatic network. Diaphragmatic lymph formation is sustained by net hydraulic pressure gradients (1, 37) driving the entrance of fluid from the diaphragmatic interstitial space and from the pleural cavity into, respectively, the initial lymphatics and mesothelial stomata (28, 31) in continuity with submesothelial lacunae (13, 20). Subsequent lymph propulsion relies on the presence of hydraulic pressure gradients developing between two consecutive tracts of the lymphatic vessel, separated by unidirectional intraluminal valves. It has been shown that the pressure gradients necessary for both formation and propulsion of diaphragmatic lymph may result from forces arising in the tissue and transmitted to the vessel lumen during the cardiac (30) and/or respiratory cycle (22). In addition, unlike what was observed in linear vessels of the central tendineous diaphragm, several loops and short linear tracts located at the outer diaphragm muscular periphery display an intrinsic pumping activity.

We have previously reported in in vivo experiments (23, 24) how each of these two pumping mechanisms, taken individually, may shape diaphragmatic lymph flux. The target of the present study was thus to attempt a more integrated description of the functional interactions between intrinsic and extrinsic, tissue-dependent, propulsive mechanisms at the level of a single loop or vessel and of the eventual modulatory interplay between the two mechanisms. To achieve this target, we developed an ex vivo experimental paradigm, i.e., the only feasible approach allowing us to neatly observe, through a conventional upright microscope at its highest magnification, the motion of single microspheres within a diaphragmatic lymph vessel experiencing simultaneous contraction of pacemaking lymphatic smooth muscles and of diaphragmatic skeletal muscle fibers. Data obtained in the present work show for the first time 1) how different the lymph flow may be, in a given diaphragmatic lymphatic structure, if supported by the spontaneous contraction of intrinsic lymphatic smooth muscle cells or by local tissue stresses; and 2) the possible interplay between these two mechanisms and which one is likely to support most of lymph transport in the normal diaphragm.

Glossary

<table>
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<tr>
<td>$J_e$</td>
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MATERIALS AND METHODS

Surgical procedures. Experiments were performed on seven healthy adult Wistar rats of both sexes (mean body weight = 404 ± 63 g). Data come from the best nine pleural submesothelial diaphragmatic lymphatic networks in which it has been possible to obtain measures during both intrinsic and extrinsic contraction. The experimental protocol was submitted to and approved by the institutional Ethical Committee for Animal Research of the University of Insubria and by the Italian Health Ministry in accordance with the 116/92 Italian law and the animal welfare directory 86/609 of the European Union.

Animals were anesthetized with an intraperitoneal injection of an anesthetic cocktail of 75 mg/kg body wt ketamine and 0.5 mg/kg body wt of medetomidine in saline solution. During the experiment, additional half boluses of ketamine were administered every 60 min, while repeatedly checking the appropriate level of deep anesthesia from the absence of the noxious hindpaw reflex.

A stable long-term fluorescent labeling of diaphragmatic lymphatics was obtained by an in vivo staining technique, similarly to what was previously reported (23, 24). Briefly, 0.8 ml of saline solution containing 1% FITC-dextran (Ex/Em: 505/515, F2505S; Sigma-Aldrich) mixed with tetramethylrhodamine isothiocyanate (TRITC)-fluorescent microspheres (500 μg/ml of injected solution, microspheres diameter: 1 μm, Ex/Em: 580/605, F-8821; Invitrogen) were injected intraperitoneally through a stainless steel cannula (outer diameter: 0.8 mm) carefully inserted through the lateral wall of the abdomen and positioned in the subdiaphragmatic medial region.

Animals were then placed prone on a warmed blanket (37°C) and let breathe spontaneously for 60 min after fluorescent dye injection. Thereafter rats were tracheotomized and intubated with a T-shaped cannula. After paralysis, induced with 0.3 ml of 2 mg/ml pancuronium bromide in saline solution delivered into the exposed right jugular vein, animals were mechanically ventilated (Inspira; Harvard Apparatus) with room air at a tidal volume and respiratory frequency automatically set by the ventilator on the basis of the individual rat body weight. The chest wall was opened exposing the pleural diaphragmatic dome, and the FITC-stained lymphatic network was visualized under a stereomicroscope (Zeiss SV11, lens 1×) equipped with a fluorescence light emitting diode (LED) epi-illuminator.

From each animal, 3 to 4 specimens of diaphragm containing spontaneously contracting lymphatic vessels were carefully excised and used in the ex vivo experiments. Depending upon the age and dimension of the rat, the diaphragm strip was 35–40 mm in length and 5–10 mm in width at its widest side close to the costal margin. The specimens, mainly belonging to the muscular periphery, consisted in thin trapezoidal muscular strips, with their major axis longitudinally oriented with respect to the skeletal muscle fibers and extending from the central tendinous region to the peripheral thoracic/abdominal wall, including a small piece of thorax to preserve the integrity of the largest number of skeletal muscle fibers (9, 17). During the whole open-chest procedure, warm (37°C) saline solution was repeatedly flushed on the diaphragmatic surface to avoid tissue dehydration. After tissue samples excision the rats were euthanized by an anesthetic cocktail overdose.

Image acquisition. Each excised diaphragmatic sample was pinned down to the bottom of a microscope perfusion chamber (RC-27D; Warner Instruments) at the same dimensions recorded in situ, leaving the tendinous edge of the specimens unconstrained. The chamber, filled with oxygenated Hepes-based Tyrode’s solution containing (in mM) 119 NaCl; 5 KCl; 25 HEPES buffer; 2 MgCl2; 2 CaCl2; and 16 D-glucose (pH = 7.4) kept at 37°C, was then placed onto the stage of an upright microscope (BX51WI; Olympus), and fluorescent dye filled lymphatics were visualized in epi-fluorescence with a dry 4× Olympus Plan APO objective (numerical aperture = 0.13). The microscope was equipped with a black and white Waterecam (WAT-902H) and connected to a personal computer running VirtualDub software (a free video acquisition and editing software; http://www.virtualdub.org/), allowing a 10 fps video collection for subsequent offline analysis.

After a conditioning period of 3–5 min, video recordings of the lymphatic network in the ex vivo tissue sample were acquired under baseline conditions for at least 1 min by alternatively exciting FITC- or TRITC-fluorophores to record, respectively, lymphatic vessels diameter (Fig. 1A) and microsphere progression along the vessels (Fig. 1B).

Diaphragmatic skeletal muscle contraction. After baseline recordings and while the lymphatic network was continuously video recorded, the striated diaphragmatic muscle fibers were electrically stimulated with trains of square electrical stimuli (200 μs pulse duration, 100 Hz pulse frequency, and 70 V amplitude) to obtain their maximal isotonic muscular contraction. The impulses were generated by a S4K Grass stimulator (Grass Instruments) and delivered in whole field configuration through two platinum wire electrodes mounted parallel on each side of the diaphragmatic specimen inside the perfusion chamber. Changes of the length of the diaphragmatic skeletal muscle fibers during maximal contraction and after complete postcontractile recovery with respect to their baseline value were obtained offline by measuring the displacement of reference points, identified

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<td>$v_{e,i}$</td>
<td>Average microspheres forward velocity (μm/s)</td>
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<td>$v_{i,max}$</td>
<td>Mean of maximal instantaneous microspheres velocity (used for Reynolds number calculus) (m/s)</td>
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on the surface of single skeletal muscle fibers under precontractile baseline condition.

**Lymphatic diameter and cross-sectional area.** Images of FITC-dextran filled lymphatic vessels were converted to black and white binary images and analyzed offline by using the automatic Diameter plugin (10) of ImageJ Software (38), to precisely determine the changes in vessel diameter encountered during spontaneous contraction of the smooth lymphatic muscles in the vessel wall and/or during contraction of the diaphragmatic skeletal muscle fibers. Corresponding cross-sectional areas were computed as \( A = [(d/2)-(d/2 \cdot 0.35)] \pi \), where \( d/2 \) and \( [(d/2)-(d/2 \cdot 0.35)] \) are, respectively, the lymphatic vessel diameter as projected onto a plane parallel and perpendicular to the pleural diaphragmatic surface. The elliptical shape with a 0.35 ratio between diameters was assumed based on functional measurements obtained in in situ diaphragm (18, 19). Because efforts were made to fix the diaphragmatic samples in the ex vivo set-up maintaining the pre-excision size, we expect such diameter ratio to still be valid for the ex vivo lymphatic vessels in the ex vivo experiments.

The Standard Deviation Z-project plugin of ImageJ Software was applied to short image sequences of complete intrinsic contraction cycles to identify, along the vessels, the intrinsic pumping sites, which, being the sites characterized by the sharpest diameter change, are highlighted by the software plugin as the thickest region of the vessel rims.

**Microspheres pathways in lymphatic vessels.** Images of TRITC-labeled microspheres moving along the lymphatic vessels were analyzed to measure microspheres progression (in \( \mu m \)), velocity (in \( \mu m/s \)), and the corresponding lymphatic flux (in \( nl/min \)) during either intrinsic pumping of the vessel and/or contraction of skeletal muscle fibers. Video-recorded images of intrinsically pumping vessels were grouped based on the direction of microsphere progression with respect to valve location, and for each vessel three to four successive intrinsically contracting cycles were examined. To define instantaneous progressions (in \( \mu m \)) at each intrinsic stroke, the distances covered by at least 10 microspheres within the vessel in 0.5- to 1.0-s intervals were measured by tracing microsphere trails obtained by the Maximum Projection plugin of ImageJ Software applied to short image sequences. The microspheres instantaneous progressions were then divided by the elapsed time to calculate the microspheres instantaneous velocity (\( \nu^{i,s} \)). Similar measurements were performed to evaluate the instantaneous progressions at each extrinsic contraction-dependent microsphere movements.

**Intraluminal lymph fluxes.** On the assumption that microsphere displacement is purely convective within the vessel, instantaneous intrinsic (\( J_{i}^{*} \)) and extrinsic (\( J_{e}^{*} \)) lymph fluxes were calculated as \( J_{i}^{*} = V^{i} \cdot A \) and \( J_{e}^{*} = V^{e} \cdot A \), where \( V^{i} \) and \( V^{e} \) are the instantaneous microspheres velocity associated with intrinsic and extrinsic contraction, respectively, and \( A \) is the lymphatic cross-sectional area measured during contraction (intrinsic or extrinsic). Intrinsic \( J_{i}^{*} \), and extrinsic \( J_{e}^{*} \), were then integrated by Clampfit 10 Software (Molecular Devices) and divided by the time period to calculate mean lymph flow rates (\( J_{i} \), intrinsic; \( J_{e} \), extrinsic). To assess the laminarity of \( J_{i}^{*} \), and \( J_{e}^{*} \), the Reynold's number was calculated as \( [Re = (d \cdot \delta \cdot \nu^{max})/\eta] \), where \( \nu^{max} \) is the mean of the maximal instantaneous lymph velocities (in \( m/s \)), \( d \) the vessel diameter during systole (for intrinsic pumping) or during maximal skeletal muscle contraction (in \( m \)), and \( \delta \) and \( \eta \) are the lymph density [0.997 Kg/m3] and viscosity [0.0009 Kg/(m*s)] (33), respectively.

**Statistical analysis.** All data are expressed as means ± 1 SE. Significance between mean values was tested by paired or unpaired Student’s t-test after data normality check. Statistics were performed with SigmaPlot 10.0 software (Systat Software).

**RESULTS**

All lymphatic vessels investigated in the present work displayed an intrinsic spontaneous contractility coupled with their perpendicular orientation with respect to the diaphragmatic skeletal muscle fibers. Because skeletal muscle contraction causes shrinkage of perpendicularly oriented lymphatic vessels (23), these vessel properties enabled us to compare the effect of the intrinsic or extrinsic mechanisms on the reduction of the lymphatic vessel diameter in the very same vessel segment.

During spontaneous contractions, not the whole vessel but only spatially confined segments displayed rhythmic contractions. As an example, in vessel of Fig. 2A, the active domain, identified from the visual representation of the standard deviation of the vessel walls displacements shown in Fig. 2B, was limited to segment \( b \) (and white asterisks in Fig. 2B), as also confirmed over time by vessel diameter that cyclically decreased at site \( b \) (Fig. 2C, trace b), but not at adjacent noncontracting sites \( a \) and \( c \) (Fig. 2C, traces a and c).

**Intraluminal microsphere movements in spontaneously contracting lymphatic vessels.** Although the actively contracting sites were discrete and occupied a limited segment of the whole vessel length, their activity was able to sustain a significant and complex motion of intraluminal lymph and microspheres. This phenomenon may be appreciated in the vessel of Fig. 3A (FITC-dextran fluorescence), by observing the position of the single microspheres (black dots) when there is no motion (Fig. 3B) and the path traveled by each microsphere, obtained as the maximum projection of the image sequence during a time frame of 0.3 s, when the vessel segment spontaneously contracts (Fig. 3C). In segments with spontaneous contractile activity (\( n = 9 \)) the mean diastolic diameter was 157.1 ± 31.8 \( \mu m \) and decreased to 133.4 ± 27.5 \( \mu m \) (85.1 ± 1.8% of the diastolic value) during systole, with a 27.3 ± 3.2% reduction of the average cross-sectional area. The contraction frequency was 20.6 ± 1.5 cycles/min corresponding to
a contractile cycle of 3.0 ± 0.2 s, of which 32.1 ± 2.0% spent in systole and 67.9 ± 2.0% in diastole.

Intraluminal microspheres during spontaneous contractions of vessel segments showed a forward and a reverse phase of motion, assessed ex post, being defined forward the flow direction observed in the same vessel during contraction of the diaphragmatic skeletal muscle. During the forward phase, which in all vessels tested corresponded to the systolic phase of intrinsic contraction, microspheres travelled by 87.4 ± 14.1 μm, whereas in the subsequent diastolic phase microspheres moved back by 75.1 ± 11.2 μm (P < 0.05, n = 9, paired t-test; Fig. 4A and Supplemental video 1), resulting in a net forward progression of 14.1 ± 2.9 μm for each intrinsic contraction cycle (Fig. 4C, light gray bar). Average instantaneous microsphere velocity (v*max) was significantly faster (P < 0.05, paired t-test, n = 9) in the forward (53.6 ± 9.1 μm/s) than in the reverse (36.7 ± 7.5 μm/s) direction.

Induction of diaphragmatic skeletal muscle contractions and associated lymphatic intraluminal microsphere movements. Whole field electrical stimulation of the diaphragmatic specimen caused a significant skeletal muscle fibers shortening, with respect to their precontractile resting length, of 24.1 ± 3.2% (P < 0.01 vs. 0%, t-test, n = 11). On average, contraction and subsequent relaxation lasted 1.2 ± 0.3 s and 6.2 ± 0.1 s (n = 11), respectively. Active contraction of skeletal muscle fibers elicited, compared with what was observed in spontaneous contraction, a faster propulsion of the lymph, as clearly indicated by the longer trails followed by the single microspheres in the example of Fig. 3D. During skeletal muscle fibers contraction, lymphatic vessels diameter decreased to 107.6 ± 23.7 μm (67.6 ± 2.2% of the diastolic value), i.e., significantly (P < 0.01, n = 9, paired t-test) more than what observed during intrinsic contraction. Meanwhile, microspheres were pushed forward by 441.9 ± 159.2 μm (range, 56.9 to 1085.2 μm; Fig. 4B), a value significantly higher (Fig. 4C, P < 0.05, paired t-test, n = 9) than those attained during forward-reverse intrinsic motion.

Comparison of forward velocities during intrinsic and extrinsic contractions. Figure 5A presents the plot of microspheres v*e,i in a vessel segment subject to two cycles (lasting 9.8 s) of spontaneous contraction followed by an induced skeletal muscle contraction (lasting 3.9 s). The time integral of v*e,i (Fig. 5B) provides the mean distance traveled by the microspheres, which, divided by the average cycle time period, allowed to calculate the average microspheres forward velocity (v*e). The latter appears to be much higher (Fig. 5C, P < 0.05, paired t-test, n = 9) when lymph is propelled by skeletal muscle contraction (v*e = 339.9 ± 122.7 μm/s) than by spontaneous lymphatic smooth muscle cell contractility (v*i = 17.9 ± 3.6 μm/s; n = 9), with no apparent relationship with vessel diameter, position over the diaphragm or vessel length.

Microspheres pathways in lymphatic vessels. Figure 5D presents the v*e,i observed in six lymphatic segments during either the intrinsic or extrinsic contraction, respectively, plotted against their corresponding radial distance expressed as distance from the longitudinal vessel axis. To compare the values obtained in different vessels, v*e,i were first normalized with respect to that (set at 100%) of the microspheres running at 0 ± 6% from the central longitudinal vessel axis. Percentual v*e,i and distances from the vessel center were then multiplied by corresponding absolute mean values to obtain the actual values presented in Fig. 6. The scatter plots referring to the intrinsically and extrinsically sustained contractions were described by the parabolic equation: v*e,i(r) = v*max·[1 - (r^2/R^2)], where v*e,i(r) is the instantaneous velocity at distance r from the longitudinal axis of the vessel and v*max represents the maximal instantaneous flow velocity at the central vessel axis. After fixing v*max and the radius R, i.e., the measured values, the fitting calculated the v*e,i corresponding to each radius in the
defined range. The equation parameters are reported Table 1, which also presents the average hydraulic radius extrapolated from the parabolic fitting and the Reynold’s number calculated at the fastest instantaneous velocity of microspheres’ forward motion during intrinsic and extrinsic muscle contraction. Both the parabolic fitting and the low Reynold’s number confirmed the laminar nature of the flow regime.

Reynold’s number calculated for the fastest $v^*$, during forward motion of microspheres induced by intrinsic pumping activity (see METHODS for calculus details) was equal to $8.1 \times 10^{-5}$, and it increased to $9.6 \times 10^{-2}$ during extrinsic contraction ($v^*$).

The shear stress $\tau$ (Table 1) was calculated as: $\tau = \mu \left( \frac{\partial u}{\partial r} \right)$, where $\mu$ is the fluid kinematic viscosity (0.009 dyne s cm$^{-2}$) taken from Ref. 33 and $\partial u/\partial r$ is the shear rate (s$^{-1}$) calculated from the parabolic fit considering both hydraulic or actual vessel radii.

Lymph flux during intrinsic and extrinsic contraction. Figure 6 shows examples of instantaneous flow rates set by intrinsic pumping alone (Fig. 6A) and by a single skeletal muscle contraction (Fig. 6B; asterisk indicates the time period of the application of the electrical stimulus) superimposed to intrinsic spontaneous contractions. Mean lymph flow rates averaged over a time period of 30 s for a single intrinsic contraction and of 1.3 s (i.e., the expected inspiratory period based on a rat resting respiratory rate of 46.7 breaths/min) (15, 21) for skeletal muscle stimulation were dramatically different, with $J_1 = 4.0 \pm 1.4$ nl/min and $J_2 = 39.8 \pm 11.4$ nl/min ($P < 0.05; n = 9$, paired $t$-test; Fig. 6C), respectively. The overall complex motion pattern of the lymph can be appreciated in Fig. 8, showing how the relative distance traveled by microspheres in a single contracting vessel endowed with lymphatic smooth muscle sharply increases in correspondence with every electrical stimulation to the skeletal diaphragmatic muscle fibers (asterisks in Fig. 7), suggesting that this latter contribution is the most important in providing forward lymph motion.

From the trace of Fig. 6B, it is not clear to what extent the whole-field electrical stimulation (needed to induce skeletal muscle contraction) interfered with the pacemaking activity of the lymphatic smooth muscle cells. To check this point out, we stimulated a diaphragmatic tissue sample to fatigue, up to the point when stimulations did not elicit any further skeletal muscle twitch; in these conditions any change in lymphatic vessel diameter and, therefore, any intraluminal lymphatic flow could be attributed only to the contraction of smooth muscle cells. As evident from the behavior of $J^*$ over time in Fig. 8A, during a prolonged (17 s) electrical stimulation of the fatigued diaphragm the intrinsic contraction was not abolished, although it appeared to progressively wane off. However, as indicated by the change in slope of the relative distance over time plot of Fig. 8B, a sustained electrical stimulation caused an evident progressive decrease of both forward and, in particular, reverse displacement, resulting in an increase of net forward motion of microspheres. This phenomenon is accompanied by the statistically significant shortening ($P < 0.01$; Student’s $t$-test; $n = 6$ measures for each trace) of time period of the flux (Fig. 8C) and relative distance (Fig. 8D) waves observed during electrical stimulation (dashed lines; period: $1.4 \pm 0.02$ s) compared with prestimulus baseline (solid lines; period: $1.6 \pm 0.01$ s).

**DISCUSSION**

**Advantages and limits of the ex vivo experimental set up.**

The use of the ex vivo approach in the present study has brought several advantages over the in vivo paradigm. Indeed, to infer on the lymphatic intraluminal fluid dynamics of lymph when propelled by contraction of lymphatic smooth muscles in the vessel wall or by the external skeletal muscle fibers in the very same vessel revealed not possible in the in vivo, in situ approach because, first, in the diaphragm intrinsically contractile vessels are very few and confined in the most peripheral muscular portion near the outer thoracic rim. Due to the specific anatomical locations of these vessels and the physiological curvature of the diaphragmatic dome in this apposition zone with the chest, it is not possible to appropriately visualize in focus in situ images of the entire lymphatic vessel. Second, such a major drawback worsens when the wall of the vessel intrinsically contracts or, even more, when the diaphragmatic muscle fibers are physiologically activated through stimulation of the phrenic nerve, which causes abdominal displacement and change in shape of the entire diaphragm with a wide displacements of the visualized field. Third, alternative approach to observe the in vivo, in situ peripheral lymphatic

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**Fig. 4.** A: plot of forward and reverse distances traveled by microspheres during intrinsic contractile cycles. $*P < 0.05$ vs. forward progression. B: plot of forward and reverse distances traveled by microspheres during extrinsic contraction, where a forward-only movement has always been recorded. C: plot of net forward distance traveled by microspheres during intrinsic (gray bar) and extrinsic (dark bar) contraction. $*P < 0.05$ vs. net intrinsic progression.
vessels would be though stereomicroscope imaging, whose low magnification does not, however, allow an acceptable optical resolution of the intraluminar microspheres used as a tool to acquire the lymph fluid dynamics parameters.

Hence, to overcome these experimental flaws and limit tissue displacement, we removed the anatomical constraints, excising a wide portion the diaphragm around the visualized intrinsically contracting lymph vessel and electrically stimulating the diaphragmatic sample in a perfusion chamber. With this approach it was possible to recruit and activate all muscle fibers around the observed vessel in a reproducible fashion.

Although potential length shrinkage upon excising was limited as much as possible by fixing the excised diaphragm tissue samples at their original in vivo dimensions, the present ex vivo preparation is far from mimicking the behavior of the entire diaphragmatic lymphatic network when normally exposed to activation via the phrenic nerve. Indeed, the physiological inspiratory diaphragmatic contraction occurs through gradual muscle fiber recruitment, depending upon the breathing pattern and inspiratory effort, and simultaneous stretching of the central tendon upon shortening of the contracting peripheral muscle fibers. Accordingly, the diaphragmatic lymphatics physiologically undergo opposite tissue stresses during the respiratory cycle, depending upon their specific anatomic location and path in the diaphragm. However, the target of this study was not to model the flow dynamic through the entire diaphragmatic mesh but, rather, to compare the impact of lymphatic or skeletal muscles activation on the flow pattern in the very same vessel. In any case, we tried to preserve as much as possible the physiological tissue environment of the investigated lymphatic vessels by excising roughly trapezoidal diaphragmatic specimens several millimeters wide, which included the highest possible number of intact muscle fibers around the lymphatic vessel chosen for the analysis. Such a wide dissection strategy offered twofold advantages: 1) the lymphatic vessel under investigation was placed in the center of a wide tissue portion, so the mechanical stresses applied to the vessel during surrounding skeletal muscle contraction ought to mimic the distribution and the amplitude of those experienced in situ, and 2) preserving whole-length, undamaged skeletal muscle fibers around the vessel assures their proper contraction when the tissue specimen is subjected to

Fig. 5. A: instantaneous velocity profile vs. time of microspheres in a single vessel acquired during spontaneous contractions and during a single skeletal muscle contraction starting at 13.3 s. Dashed lines indicate the zero crossing reference points for the boundaries of the integration periods, which give rise to the integrals plotted in B. B: time integrals of instantaneous velocities calculated from the trace of A during intrinsic (gray area) and extrinsic (black area) contraction. Integrating limits encompass 2 cycles for intrinsic contraction and 1 single skeletal muscle contraction for extrinsic contraction. They start with zero instantaneous velocities and end when the instantaneous velocity has returned to zero (dashed lines mark these reference points). C: comparison of mean microsphere velocity due to intrinsic (gray bar) or extrinsic (dark bar) contractile mechanisms. Mean values are obtained by dividing the value of the time integral (as the example of B) at the end of the indicated integrating period (9.8 s and 3.9 s for intrinsic and extrinsic propulsive actions, respectively, in the example reported in B) by the integrating period itself. In the example reported in A and B, mean velocity was 15.4 μm/s during intrinsic contraction and 70.2 μm/s during skeletal muscle contraction, respectively. *P < 0.05 vs. intrinsic propulsion. D: plot of instantaneous microsphere velocity as a function of its position relative to the central longitudinal axis of the lymphatic vessels during intrinsic (hollow circles) or extrinsic (solid circles) contraction. Dotted and dashed lines represent the parabolic profile fits to the 2 sets of data points (see RESULTS for fitting equations). Vertical small dotted line indicates vessel center.
whole-field electrical stimulation (17). Explanted oxygenated diaphragmatic specimens kept at 37°C were allowed to preserve the original contractile properties of the skeletal diaphragmatic (9) and of lymphatic smooth muscles. Indeed, on one hand lymphatic vessels maintained their prolonged and sustained intrinsic contractility throughout the whole duration of recordings, and, on the other, electrical stimulation of the skeletal muscle, albeit able to elicit a maximal contraction, caused a limited movement of the observed field, allowing an easy resolution and recording of intraluminal single microspheres and aggregates (Fig. 1B) even with a 4× objective (field of view of 1.1 mm in diagonal) in an upright microscope. Hence, our ex vivo approach has broadened the spectrum of mechanical stimuli that can be applied to the diaphragmatic lymphatic vasculature while simultaneously recording several functional parameters otherwise not collectable in the in vivo, in situ preparation (17). In fact, the mean muscle fiber shortening (∼24% of precontractile length) attained with whole-field electrical stimulation, similar to that observed when contractions are elicited by KCl injections in the interstitial space among muscle fibers (23), is in good agreement with that expected in the diaphragm during spontaneous inspirations (4, 7, 42).

**Lymph motion during spontaneous intrinsic contraction.** Despite the presence of scattered intraluminal valves (23), the propelling efficacy of the spontaneous contraction alone in terms of net lymph displacement was hampered by the fact that most of the forward displacement during the systolic phase was counterbalanced by a backward flow in the subsequent diastolic phase (Fig. 4A and Supplemental Video 1). Indeed, in these diaphragmatic lymph vessels intraluminal valves, especially if located outside the contracting portion of the vessel, were biased in an apparently open position, a phenomenon also observed in mesenteric lymphatics (2, 5) so that microspheres easily travel along the valve in both directions. As a result, the net $\bar{v}_i$ is as low as ∼18 μm/s (∼30% of the $v^s$, during the systolic phase), confirming the previous observation that fluid flux is much slower in these lymphatic vessels located at the extreme muscular periphery of the diaphragm than in those running more medially toward the tendinous portion of the diaphragm (18). Hence, the overall indication of the present results is that, at variance with what is commonly observed in other spontaneously contracting lymphatic collectors (43) in peripheral diaphragmatic lymphatics, the intrinsic contractility plays a minor role compared with that of skeletal muscle contraction (Fig. 5). A comment is due, however, on the fact that we cannot exclude that the explants procedure might have impaired the contractile properties of the vessels segments under study, thereby also diminishing the estimated relative contribution of the intrinsic versus the extrinsic mechanism. Indeed, in ex vivo spontaneous contraction, vessel diameter reduced to ∼85%, of diastolic diameter, i.e., much less than what was observed in spontaneously contracting vessels in, in situ open chest diaphragm (24), but similar to that encountered in diaphragmatic vessel segments with a distension-triggered active contraction (24). Because we were unable to distinguish between the two types of contractile segments, we cannot attribute the present result to a prevalence of distension-triggered vessels or to other differences between the in vivo and ex vivo preparation, such as the differences in the curvature of the diaphragm or the direction of applied stresses.

Another important parameter to be taken into account is transluminal pressure and imposed flow that have been shown to affect lymphatic flow dynamics.

### Table 1. Data (from 6 vessels) describing the flow dynamics attained during both intrinsic and extrinsic propulsion in peripheral diaphragmatic lymphatics

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<td>$T_s$, dyne/cm²</td>
<td>0.02 ± 0.006</td>
<td>0.23 ± 0.08*</td>
</tr>
<tr>
<td>$R_s$, μm</td>
<td>87.3 ± 17.8 §§</td>
<td>84.1 ± 17.1* §§</td>
</tr>
<tr>
<td>$T_h$, dyne/cm²</td>
<td>0.02 ± 0.007</td>
<td>0.25 ± 0.09</td>
</tr>
<tr>
<td>$T_r$, range</td>
<td>0.001 ± 0.05</td>
<td>0.02 ± 0.55</td>
</tr>
<tr>
<td>$R_r$, range</td>
<td>0.001 ± 0.05</td>
<td>0.020 ± 0.59</td>
</tr>
<tr>
<td>$R_w$</td>
<td>8.1·10⁻¹</td>
<td>9.6·10⁻²</td>
</tr>
</tbody>
</table>

Values are means ± SE. The flow velocity profile has been described through a parabolic equation, and the fitting parameters have been averaged to give the presented mean values. $V_{max}$, measured highest velocity; $V_{max-fit}$, highest velocity from parabolic fit of the data; $R_v$, real vessel radius, measured; $T_s$, wall shear stress computed at the real vessel radius; $R_s$, hydraulic radius from parabolic data fitting; $T_h$, wall shear stress computed at the hydraulic radius; $R_w$, Reynolds’ number. *P < 0.05, **P < 0.01 vs. intrinsic, paired t-test; $tP$ < 0.05, §§§P < 0.01 fit vs. corresponding real value, paired t-test.
to significantly affect spontaneous contractility in mesenteric lymphatics (11, 35). In peripheral diaphragmatic lymphatics of paralyzed oxygenated rabbits with closed chest, at a lung volume corresponding to functional residual capacity, transmural distending pressure, calculated as the difference between intrapleural fluid pressure and intraluminal lymphatic pressure, was ~0.5 cmH₂O, a value similar to that obtained in the same animal when opening the chest (27). Transmural pressure likely increases during inspiration when intrapleural pressure further decreases; hence, provided the contractile diaphragmatic lymphatics display a transmural pressure dependence as other lymphatics do, one would expect the contribution of the intrinsic mechanism to increase under spontaneous breathing. Yet, functional data indicate that amiloride inhibition of contractility of pleural lymphatics reduced pleural lymph drainage by about 40% (26), suggesting that, unlike what observed in most tissues, pleural lymphatic function is mostly supported by transmural pressure oscillations generated by tissue displacement and stresses. The difference in the relative importance of the two mechanisms in the diaphragm compared with other tissues might be due to the uneven and irregular distribution of α-actin and, therefore, smooth muscle cells in the wall of the diaphragmatic peripheral vessels (24) compared with other districts, such as the mesentery, where lymphatics present a more ordered and continuous chain of contracting lymphangions.

On considering that the intrinsic mechanism is reduced or even stopped with vessel overdistention due to an increase in inlet pressure or imposed flow (11, 35), one might expect the prevalence of the extrinsic over the intrinsic mechanism to progressively increase with increasing fluid drainage, like, for example, in case of pleural effusion. Being that this is true also for the lung and with the consideration that passive mechanical ventilation without active inspiratory muscle contraction inhibits lymph flow from thoracic tissues (22), maintenance of

![Fig. 7.](image-url) Fig. 7. Plot of relative distance traveled by microspheres in a vessel segment during intrinsic and superimposed extrinsic contraction. *Time of application of 2-s electrical stimulations to the diaphragmatic specimen.

![Fig. 8.](image-url) Fig. 8. A: plot of instantaneous flux vs. time during intrinsic contraction in a lymphatic vessel hosted in a diaphragmatic specimen whose skeletal muscle has been brought to fatigue by means of repetitive induced contractions. A prolonged (17 s, solid horizontal line) electrical stimulation of the diaphragmatic muscle did not stop the lymph flow due to intrinsic contractility. B: plot of relative distance traveled by microspheres before, during, and after the prolonged electrical stimulation (solid horizontal line, as in A). C: superimposition of the trace showing the instantaneous flux measured during the electrical stimulation (dashed line) and the 1 measured before that, spanning an equivalent time period (solid line). D: superimposition of the trace showing the relative distance traveled by microspheres during the electrical stimulation (dashed line) and the 1 measured before that, spanning an equivalent time period (solid line). For both C and D, period of dashed trace is significantly shorter if compared with the 1 of solid trace (1.4 ± 0.02 s vs. 1.6 ± 0.01 s; *P* < 0.01, *t*-test; *n* = 6 measures for each trace).
spontaneous ventilation would be crucial in preventing and/or limiting the occurrence of pulmonary edema.

Lymph motion upon contraction of skeletal diaphragmatic fibers. When a given vessel was exposed to contraction of diaphragmatic skeletal muscle, intraluminal flow pattern invariably and dramatically changed with respect to what observed during spontaneous contraction alone: indeed, lymph progression became unidirectional (Fig. 4B, ~440 μm displacement/stroke), with no reverse flow and a v*e > 300 μm/s (Fig. 5C). In addition to the significant increase in velocity, likely due to the higher power stroke and squeezing effect exerted on the vessel wall by contracting skeletal muscle fibers, the most striking observation was that despite vessels that returned to their resting diameter upon muscle relaxation, no reverse flow has ever been detected. This phenomenon may be justified by the fact that because skeletal muscle contraction squeezed the vessel along its whole length and with a greater force than that exerted on a single segment during spontaneous local contraction of smooth muscle cells in the lymphatic wall, a much larger volume of lymph was propelled.

Forward flow velocity during extrinsic pumping is affected by a great variability, which does not depend by vessel geometry or direction but might more reasonably depend upon the actual force exerted by the contracting skeletal muscle. In the present experiments contraction was evoked in almost zero load conditions in the unconstrained diaphragm. In this condition the contraction velocity approaches Vmax and the force load conditions in the unconstrained diaphragm. In this condition, when diaphragmatic fibers are held at a length closer to their resting one by the thoracic and crural physiological anatomical connection. The contractility velocity of the skeletal muscle fibers and the forces they actually exert are highly in-homogeneously distributed within the diaphragm (6, 42), so their impact on local lymph flow is expected to be very complex and depending upon 1) the anatomical position of lymph vessels with respect to muscle fiber direction (23), 2) their location with respect to the muscular and tendineous portion of the diaphragm, 3) their anatomical origin, i.e., whether the lymph vessel drains from the pleural or the peritoneal cavity, and last but not less important, 4) the extent of the number of recruited diaphragmatic fibers and their contractile force. A detailed analysis of these factors is beyond the scope of the present work but it will be required for a better comprehension of the lymph flow setting and modulation in the diaphragm.

Shear stress and intraluminal valves configuration. The parabolic profile of the v*i.e (Fig. 5D) and the low Reynold’s numbers (Table 1) characterizing lymph flow sustained by both spontaneous and skeletal muscle contractions witness the occurrence, in both cases, of a laminar flow regime. Shear stress values calculated at the vessel wall (see Table 1) are almost two orders of magnitude lower than those obtained in mesenteric or dermal lymphatics (2, 8, 36), and very close to a previous indirect calculation on diaphragmatic lymphatics (18). Moreover, because actual lymph vessel cross section is elliptical, with a 0.35 ratio between diameters (18, 19), the actual viscous flow resistance is higher than that predicted by the Poiseuille’s law alone by a multiplying factor C defined as (25): C = (P/2A), where p is the wetted perimeter and A the vessel cross-sectional area. For an average vessel used in the present study (major diameter = 157 μm), C = 18.3, so the actual viscous forces would be ~18 times higher than those predicted for a circular vessel section. Assumption of the 0.35 ratio was made as a first approximation for our model on the premises that such ratio had not changed, or if any had been only slightly modified, than that measured in the in vivo, open chest diaphragm (18). Indeed, in the present ex vivo preparation a large surface of diaphragmatic tissue around the vessels to be studied was excised, so that the reciprocal connections with the surrounding tissue and the orientation of the vessel within the tissue were not changed or, if any, only slightly modified. Because of the much higher v*i.e during diaphragmatic skeletal muscle than v*i, during spontaneous lymphatic smooth muscle contraction, the corresponding wall shear stress increased almost 15 times more during extrinsic pumping, likely representing an important determinant of the transition from a bidirectional to unidirectional intraluminal flow. Indeed, for very low Reynold’s numbers, intraluminal valve closure depends upon pressure drop and viscous forces alone (16); the high forward v*i.e attained during skeletal muscle contraction might rise both these parameters enough to trigger closure of the intraluminal valve thus preventing lymph backflow, and favoring unidirectional forward flow. On the contrary, the extremely low v*i.e observed with spontaneous contraction alone would not be able to set the conditions favorable to backward valve closure, thus resulting in significant backflow and slow forward propulsion. Hence, it can reasonably be expected that diaphragmatic lymphatic drainage mostly depends upon the extrinsic component of flow motion, as previously suggested based on a previous functional studies (26), and reflects the force and the frequency of skeletal muscle activations. This is in accordance with what has been found in paralyzed animals, which, in the absence of voluntary activations of the diaphragmatic muscle, displayed a less pronounced pressure gradient favouring lymph drainage (22).

Unfortunately, in our ex vivo preparation of the diaphragmatic excised tissue, due the slight motion of the tissue during electrical stimulation, no direct recording of valve position has been possible and valve open/closed configuration could be derived only by microsphere flow direction. A comment is due on the possibility that valve behavior might be modified by the ex vivo preparation. Indeed, on one hand, in the excised specimens exposed to atmospheric pressure, the lack of a subatmospheric pleural pressure distending the superficial diaphragmatic lymphatics might cause vessel collapse and greater valve continence. On the other hand, in the intact chest, lymph flow within a given diaphragmatic vessels would surely be higher than in the excised specimen, particularly at end expiration, when pleural liquid pressure is the least negative over the respiratory cycle. Hence, we may expect lymph velocity to be higher and, according to Bernoulli’s effect, lymphatic valves to be more continent than in our excised preparation. During inspiration, the further intrapleural pressure drop and to the contraction of diaphragmatic muscle fibers lymphatic vessel section ought to increase in superficial submesothelial vessels and decrease in collecting lymphatics running deeper within the contracting muscle fibers, so that valve
behavior might be different according to vessel position within the diaphragm. In any case, we believe that the indications of the present study may be considered reliable, since the intrinsic contraction dependent forward flux has been invariably detected in the very same vessel tracts where intrinsic contraction sustained a forward-reverse flow, so that it may be reasonable to assume that the change in flow pattern is secondary to the change in behavior of the valves, which switches from an always open state to a open-closed state (see Supplemental Video 1 for intraluminal valve behavior during intrinsic contraction).

Intrinsic-extrinsic interplay in lymph flow and propulsion. Increased shear stress induces the synthesis of nitric oxide from lymphatic endothelial cells and the reduction of smooth muscle tone in lymphatic smooth muscle (3, 12, 40); so, even though also intrinsic contraction depends upon fluid in the system, one might expect that intrinsic pumping might decrease during or after skeletal muscle contraction, which implies an increased flow velocity and shear stress. However, analysis of traces like the one reported in Fig. 7B shows that, unlike what observed in other lymphatic districts, such a behavior is not encountered in diaphragmatic lymphatics. On the contrary, as it is clearly evident from the analysis of the trace of Fig. 9B, the electrical stimulation is indeed able to elicit a change in the intrinsic pumping pattern, increasing net forward lymph progression. These observations can be explained based on the following considerations: 1) the shear stress is very low in these vessels and, even during extrinsic contraction, never exceeds 0.25 dyne/cm² (Table 1), a value that might be below the threshold for the induction of nitric oxide production (14, 32, 41); and 2) the source of the electrical stimulus was artificial in our experiments, and it cannot be excluded that the electrical activity of the skeletal muscle could also affect the intrinsic contraction on purely electrical basis, implying a potential electrical interplay between the two pumping mechanisms.

Concluding remarks. The use of an ex vivo approach in the study of diaphragmatic lymphatic vessels enabled us to directly compare for the first time the actual lymph flow pattern set by spontaneous contraction of the lymphatic smooth muscles or by contraction of the skeletal muscle fibers surrounding the vessel. Data analysis showed that during intrinsic pumping, albeit instantaneous velocities can be high, the forward-reverse nature of lymph flow allowed by the incomplete closure of intraluminal valves gives rise to a modest net forward propulsion of lymph, which, instead, is dramatically enhanced by the extrinsic pumping action. Shear stress values calculated from the velocity profile recorded in each lymphatic vessel are extremely low even during extrinsic contraction if compared with the values obtained in peritoneal vessels, where shear stress-mediated endothelial nitric oxide production has been largely documented. Hence, if a possible interplay between intrinsic and extrinsic contraction may occur, it has to be found in the mechanical action on intraluminal valves and on the eventual electrical interaction between skeletal muscle activation and smooth muscle, which, however, still needs to be investigated. In conclusion, although most of the forward, significant, propulsion of lymph occurs during the activation of diaphragmatic skeletal muscle, albeit at a very low rate of about 15 times/min in humans, intrinsic contractile activity in the peripheral structures keeps the lymph flowing and contributes with a modest but continuous propulsive action during the resting periods in between skeletal muscle activations, enabling a continuous lymph drainage from peritoneal and pleural cavities.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.M. and D.N. conception and design of research; A.M., E.S., and C.M. performed experiments; A.M., E.S., and C.M. analyzed data; A.M. and D.N. interpreted results of experiments; A.M. and E.S. prepared figures; A.M. and D.N. drafted manuscript; A.M., E.S., and D.N. edited and revised manuscript; A.M., E.S., C.M., and D.N. approved final version of manuscript.

REFERENCES
