# In Human Lymphatic Glycocalyx Identification by Electron Microscopy and Immunohistochemistry

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# Abstract

## BACKGROUND

Blood flow is translated into biochemical inflammatory or anti-inflammatory messages based on the type of shear stress, by means of sensitive receptors located on the endothelium. Recognition of the phenomenon is of paramount importance for the advancement in the understanding of the pathophysiological processes of vascular remodeling.

The endothelial glycocalyx is a pericellular matrix, identified in both arteries and veins, acting as sensors responsive to the flow changes. Venous and lymphatic physiology is interconnected; however, to our knowledge, a lymphatic glycocalyx-like structure has never been identified in humans. The objective of this investigation is to identify glycocalyx-like structures from *ex vivo* lymphatic human samples.

## METHODS

Lower limb vein and lymphatic vessels were harvested. The samples were analyzed by electron microscopy. The specimens were also examined by immunohistochemistry.

## RESULTS

Scanning electron microscopic identified a glycocalyx-like structure in both venous and lymphatic structures. Immunohistochemistry for podoplanin, glypican-1, mucin-2, agrin and brevican characterized both the lymphatic and venous structures.

## CONCLUSIONS

To our knowledge, the present work reports the first identification of a glycocalyx-like structure in the human lymphatic. The vasculoprotective action of the GCX could become an investigational target in the lymphatic system as well, with clinical implications for the many patients affected by lymphatic disorders.


# Introduction

The vascular endothelium has been defined as an “organ” due to its multiple influences upon vessel contraction, cellular and and nutrient trafficking, coagulation balance and angiogenesis [1].

These phenomena are associated with the interaction between the flow force and the related endothelial release of biochemical products. The translation of shear stress in to different endothelial phenotype expressions is known as “mechanochemical transduction” [2]. While laminar flow leads to anti-inflammatory endothelial expression, turbulent flow generates pro-inflammatory, pro-thrombotic and pro-atherosclerotic signaling [3]. The glycocalyx (GCX) is a multi-component structure covering the endothelial surface, and acting as an extremely sensitive receptor of the flow features. It is made by

carbohydrate chains, including membrane-bound glycoproteins, proteoglycans, and sulfated glycosaminoglycan side-chains, constituting in aggregate a negatively charged protective and dynamic layer [4]. Changes in the flow characteristics lead to alterations in the glycocalyx signaling involved with endothelial protection and permeability regulation, and leukocyte adhesion and diapedesis, as well as vessel contraction and coagulation balance modifications (platelet adherence inhibition, coagulation activation)[5].At a systemic level, the GCX demonstrated also immune and tissue healing functions [6,7]. GCX structure is altered by several pathological features, including hyperglycemia, hyperlipidemia, hypertension, trauma, thrombosis, infection, tobacco use and aging [8-10].

Atherosclerosis, stroke, and hypertension have been clearly associated with a GCX structural impairment [11]. GCX alteration also leads to dysregulation of permeability and coagulation balance, together with enhanced leukocyte adhesion, which are all aspects involved in venous disease pathophysiology [12,13]. The capillary exchange process once comprehended chiefly through the Starling principle is now known to include an active process modified by the GCX as a sieve of various porosities, thereby regulating the vessel permeability to macromolecules and, thus, implicated in eventual edema formation [14,15]. This latter observation led our research group to search for previous studies that attempted to detect the presence of the GCX in the lymphatic system.

Through an extensive search dating back to the early 1960s, when Rambourg provided evidence that the GCX was present on the surface of cells harvested from rats [16], the only paper we found on the topic is from an animal model in the murine cremaster muscles and based on confocal microscopic analysis of GCX moieties [17]. We found no publication regarding identification through *in vivo* human electron microscopic identification and immunohistochemical characterization of the lymphatic GCX, which is, therefore, the primary aim of this study.

# Materials & Methods

A lower limb vein and lymphatic sample was harvested from a 51 year-old female patient (BMI 25) undergoing a thighplasty procedure that was performed following bariatric surgery (sleeve gastrectomy). Apart from medical obesity, no significant comorbidities were reported. The patient was not a smoker.

After obtaining an informed consent, according to the Declaration of Helsinki on biomedical research involving human subjects, a periprocedural injection of patent blue was performed *for in situ* anatomic identification of the lymphatic vessels.

Ten minutes before the surgical incision, the intradermal and subcutaneous injection of 2.5 ml of patent blue was completed in two different points of the medial region of the thigh.

The excess skin was removed in agreement with the preoperative surgical planning. The excised piece was dissected outside the operating table under Loupes 3x magnification by means of microsurgical instruments, searching for the labeled subcutaneous lymphatic vessels.

Once the lymphatic vessels were identified, their lumen was washed by a delicate flush of physiological solution, followed by the dedicated fixative for electron microscopic analysis, the precise composition of which is reported in the section below. The largest needle suitable for the flush (30 G) was used G, indicating vessel diameter of approximately 0.3 mm.

After fixation, the lymphatic vessel was atraumatically removed and inserted into a vial filled with the same fixative. A vein adjacent to the harvested lymphatic was sent to the laboratory as well, following the same dissection and fixation technique (Fig 1).

The study design was approved by local institutional review board (University of Padova, N. 2658P). The study was conducted according to the criteria set by the declaration of Helsinki. The subject signed the informed consent.

### Transmission Electron Microscopy.

The specimens were divided into two fragments, one of them destined for histological analyses by light microscopy and the other one for morphological analyses by transmission electron microscopy (TEM).

For TEM analyses lymphatic vessels and veins were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), containing 0.05% (w/v) Alcian Blue 8GX, at 4°C over-night and post-fixed in 2% buffered osmium tetroxide for 1 hour. The specimens were then dehydrated with graded concentrations of acetone and embedded in Araldite epoxy resin (Durcupan ACM, Fluka, Sigma-Aldrich Co., St. Louis, MO, USA) according to standard protocols. For orientation, semi-thin sections (1.5μm) were cut on a Reichert Ultracut S ultramicrotome using glass knives and stained with a 1% aqueous solution of toluidine blue and examined with an optical microscope (Nikon Eclipse E800). Ultrathin sections (90nm) were prepared with an ultramicrotome (Reichert UltracutS) and counterstained with uranyl acetate in saturated solution and lead citrate according to Reynolds and observed under transmission electron microscope (TEM Zeiss EM 910, Zeiss, Wetzlar, Germany) at 80.000X magnification.

### Immunohistochemistry

For histological analyses, lymphatic and venous samples were fixed in formalin 10% for 24h at 4°C, dehydrated through an alcohol series and then paraffin-embedded using a Shandon Citadel 2000 Tissue Processor (Thermo Fisher Scientific, Waltham, MA). Five-μm-thick sections were cut from paraffin blocks.

The sections were stained with the primary anti-podoplanin and anti-glypican 1 antibodies, both from Abcam (Cambridge, UK), the anti-mucin-2 antibody (Thermo Fisher Scientific), and then counterstained with the anti-rabbit HRP-DAB tissue staining kit (R&D Systems, Minneapolis, MN). A negative control was obtained in each slide by carrying out the immunohistochemistry staining procedure without using the primary antibody. The images were acquired with an Aperio ScanScope® slide scanner by using the Aperio ImageScope v11.1.2.760 software (Leica Biosystems, Nussloch, Germany).

# Results

After fixation, the wall thickness of the vein and of the lymphatic samplesx measured X10 along the vessel transverse section, were 397,3±208,3 mm (min=179 mm; max=719,1 mm) and 51,3±12,6 mm (min=32,55 mm; max=71,26 mm), respectively.

TEM analysis confirmed the presence of a GCX like structure along both the vein (fig 2A) and lymphatic (fig 2B) endothelium.

The mean height of the GCX structure measured 25X along the lymphatic transverse section and 17X along the vein section was 66,94±18,38 nm (min=53,4 nm; max=113,09 nm) in the vein and 49,26±11,30 nm (min=26,61 nm; max=67,83) in the lymphatic sample ($p<0,001$). In both vein and lymphatic samples, the GCX appeared as a-filamentous-like structure, clearly distinct from the contiguous plasma membrane of the endothelium.

The vein GCX homogeneously covered the entire endothelial lining.

The lymphatic GCX layer showed interrupted zones along areas of endothelial lining disruption.

Both venous and lymphatic GCX immunohistochemistry identified glypican-1 and mucin-2.

Agrin and brevican were identified in both the vein and lymphatic samples, but on all the external surfaces of  vessels wall cells, representing a diffuse component of the extracellular matrix. Therefore, both agrin and brevican were not considered as characterizing elements of the glycocalyx.

All the results were confirmed by the negative control performed by the secondary antibody.

(Fig. 3).

Podoplanin, widely recognized as a lymphatic tissue-specific marker, was identified only in the lymphatic vessel [18].

# Discussion

GCX has been recognized as a fundamental component of the arterial and venous endothelium, involved in multiple pathophysiology processes, and therefore acknowledged as a potential therapeutic target for multiple diseases [19].

It can be visualized as a carbohydrate-rich layer anchored with proteins to the endothelium, protecting and interacting with the vessel wall, akin to like a forest sitting atop the soil.

Proteoglycans and glycoproteins represent the “trunks” anchored to the endothelium, around which soluble components from both the plasma and the same vessel wall interact in a dynamic balance with the GCX. Proteoglycans are usually comprised of a core protein attached to glycosaminoglycan chains.

Syndecans and glypicans act as core proteins. There are also glycoproteins with acidic oligosaccharides and terminal sialic acid conjugates. These are represented among the endothelial bound proteins with a polypeptide backbone that form various adhesion molecules, selectins, and integrins [4, 6, 19]. The glycosaminoglycan chains vary according to several anatomical and hemodynamic conditions. There are five sulfated glycosaminoglycan chains: heparan sulfate, dermatan sulfate, chondroitin sulfate, keratan sulfate, heparin, and one non-sulfated hyaluronic acid.

Heparan sulfate proteoglycans represent approximately 50–90% of the total GCX proteoglycan content, and, in humans, most endothelial cell glycosaminoglycans are heparan sulfate, chondroitin sulfate, and hyaluronic acid [20, 21]. The second most common is chondroitin sulfate/dermatan sulfate, usually reported in a 1 to 4 ratio when compared to heparan sulfate [22]. Additionally, the glycoproteins anchor the GCX to the endothelium and these glycoproteins include cell adhesion molecules (integrins and selectins) as well as coagulation components. The thickness, structure and electronic charge of the GCX regulates the permeability of the endothelium [23]. Being negatively charged, the GCX repels red blood cells and platelets, limiting contact with the endothelium and favoring a laminar flow [24, 25]. GCX has also beendemonstrated to regulate leukocyte adhesion to the endothelium [26]. The several components of the GCX regulate phenotypic expression of endothelial microenvironment phe, including the balance of the lipolytic [27] and anticoagulation systems [28]. Moreover, the GCX expresses a vasculo-protective role due to its ability to modulate inflammatory responses by regulating cytokine binding [29] and as quenchers of oxygen radicals [30]. In extension to the known molecular functions of the GCX, its involvement in the pathophysiology of several arterial (diabetes, ischemia, atherosclerosis) and venous (chronic venous insufficiency, venous thrombosis) diseases has been demonstrated [31–33]. Veins and lymphatics are strictly interconnected and mutually involved in lower limb fluid drainage [34]. This observation, together with the shared embryological origin of venous and lymphatic endothelium, led us to explore the eventual presence of a lymphatic GCX [35]. To our knowledge, the findings presented here are the first in human *ex vivo* identification of a lymphatic glycocalyx.

The characterization of a vein sample from the same patient offalso provides a unique opportunity to compare the GCX attributes originating from lymphatic and venous tissues, respectively.

In our observation, TEM clearly showed a GCX-like structure both along the vein and the lymphatic endothelium.

The mean thichkness of the venous GCX was higher than in the lymphatic, yet the ratio between the the GCX and the wall thickness was higher in the lymphatic segment (Table 1).

Table 1
Mean, minimum and maximum values of vein and lymphatic wall (W) and glycocalyx (GCX) thickness. GCX/W indicates the glycocalyx / wall thickness ratio.

| | VEIN | LYMPH |
|---|---|---|
| **MEAN GCX thickness (nm)** | 66,94 ± 18,38 | 49,26 ± 11,30 |
| **MIN GCX thickness (nm)** | 53,4 | 26,61 |
| **MAX GCX thickness (nm)** | 113,09 | 67,83 |
| **MEAN wall (W) thickness (mm)** | 397,3 ± 208,3 | 51,3 ± 12,6 |
| **MIN wall (W) thickness (mm)** | 179 | 32,55 |
| **MAX wall (W) thickness (mm)** | 719,1 | 71,26 |
| **GCX/W mean** | 0,02% | 0,1% |
| **GCX/W min** | 0,03% | 0,08% |
| **GCX/W max** | 0,02% | 0,1% |

This correlates with previous observations of varying GCX morphology along different anatomical locations and may be an inherent requirement of the lymphatic endothelial cells in regulating fluid and macromolecule transport from the interstitial space and in immunomodulation [36–39].

We hypothesize that a low fluid force is gently acting on the endothelium, allowing the growth of a thicker GCX compared to its counterparts covering the endothelial linings exposed to higher hemodynamic forces, where a disruptive mechanical action might operate on the delicate surface structures.

Indeed, in vein and arteries, GCX thickness has been reported to vary between 20 and 400 nm in veins and around 500 nm in arteries on average, reaching up to 4.5 micrometer in the carotid [40, 41].

The low flow in the lymphatics, when compared to the more turbulent venous flow, could explain the our current observations, if we consider the ratio between the thickness of the GCX and that of the vascular wall. Nevertheless, this hypothesis is not sustainable if the absolute thickness values are considered. Further investigations are needed on the topic, relying on vein and lymphatic samples that must be equivalent in anatomical and hemodynamic features, in order to more clearly understand these anatomic/functional relationships.

No differences between the lymphatic and venous density of the GCX filaments were demonstrable in the TEM of the patient studied, and a validated measurement method is currently lacking,; therefore, statistical analysis of this morphologic aspect of the GCX is not feasible here.

Considering the intricate mesh-like structure of the GCX and its related functionality, direct observation of its morphological and immunohistochemical features is of paramount importance.

Visualization techniques using an enzymatic wash to detect the different GCX components are essential for understanding the players components involved in the structure. Nevertheless, the complex, three dimensional architecture of the GCX requires *ex vivo* direct visualization of its entirety in order to derive proper insights.

The challenges surrounding sample fixation for microscopic analysis are well known. The GCX is an extremely fragile structure, easily altered, injured, and dehydrated during vessel handling and preparation. This has been evident already in investigations of the more robust arterial and venous structures and becomes particularly challenging in the current observations of the delicate lymphatic endothelium.

Such limitations should be taken into consideration whenever reporting the GCX dimensions, since GCX measurement following fixation could be significantly underestimated due to the loss of thickness in the fixation process [42].

In parallel with the TEM observations, our research lab performed immunohistochemical analysis for characterization ofthe vein and lymphatic components.

As expected, podoplanin was detected only in the lymphatic sample inasmuch as it is a specific lymphatic tissue marker. We rely upon anti-podoplanin staining to distinguish the lymphatic vasculature from venous and arterial vessels within the anatomic specimen [18, 43, 44].

Glypican-1 was identified in the vein GCX, correlating with previous reports regarding its function in endothelial homeostasis and, in particular, on its anti-atherosclerosis properties [45, 46].

A recent review highlighted the role of glypican-1 in regulating multiple cellular signaling pathways, including fibroblast growth factors, vascular endothelial growth factor-A, transforming growth factor-β, and bone morphogenic protein [47].

We identified mucin-2 as another component of the lymphatic GCX.

The presence of mucin within the GCX has been described for decades [48].

Interestingly, mucin-2 has more recently been demonstrated too occupy a role in preventing intercellular junction defects, s a feature to known lymphatic filtration functions [49].

We also detected brevican as another component. It is a proteoglycan previously identified in the nervous system, where it represents a key element of the peri-synaptic extracellular matrix. Its role as regulator of synaptic plasticity and perineural nets creation suggests further work is necessary to define its role in the function of the lymphatic matrix [50].

Our immunohistochemical analysis also identified agrin in the lymphatic GCX.

This is another proteoglycans recently investigated in the realm of angiogenesis regulation. Detection of agrin in the lymphatic GCX mandates further exploration of its role in lymphatic vessel development and proliferation [51–52].

However, it must be re-emphasized that both brevican and agrin were not found exclusively in the glycocalyx, rather, they also represent components of the extracellular matrix. Further investigation is needed to explore their potential role in mechanotransduction.

The findings of this study are compatible with a potential role of the GCX in lymphatic function and in the related fluid filtration.

This is a pilot study that stimulates further detailed characterization of what is, until now, the first in human identification and visualization of the lymphatic GCX.

The limitations of the present research include the single patient nature of the analysis, in the possible shedding of GCX components during harvesting and during the fixation flush, and, potentially, in the limited number of glycoproteins, proteoglycans, and glycosaminoglycans identified.

The need of an advanced assessment methodology for the GCX in vivo investigation has been recently reported by Haymet et al., addressing the need of static as well as dynamic models [53].

The translational importance of this bench investigation can be inferred from the intricate pathophysiology of diseases related to arterial, venous, and lymphatic mechanisms.

In this sense, GCX assessment has been recently introduced in the clinic with a dedicated sublingual test named “GlycoCheck” able to report the same GCX thickness [53].

Particularly in time of COVID, the role of endothelial inflammation and the importance of its counteraction has been made clear to the scientific community, while the GCX has been already identified as a potential treatment target for the microvascular endotheliopathy [54]

that according to our research is of considerable interest in the study and pathology of the lymphatic system.

# Conclusions

GCX has already been identified as a key element in arterial and venous mechano-transduction. Its role in vessel protection, permeability and contraction, together with the related coagulation balance control is involved in the pathophysiology of both arterial and vein disease [4].

The present investigation provides the first in human identification and characterization of a GCX structure also present in the lymphatic system, providing a comparative analysis of *ex vivo* samples of lymphatic and venous tissue obtained from the same subject. The study paves the way for further assessments in the different parts of the lymphatic systems, in both physiological and pathological

conditions, potentially providing translational resources that will inform the future management of the extremely relevant clinical issue of edema.

# Declarations

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**Author contributions:**

Conception and design: SG, ER, JDR, SR

Analysis and interpretation: SG, ER, JDR, SR, EM, AP

Data collection: EM, AP, GA, FB, EMEN, AC

Writing the article: SG, ER, JDR, SR, AC, FB

Critical revision of the article: SG, ER, JDR, SR, EM, AP

Final approval of the article: SG, ER, JDR, EM, AP, EMEN, GA, FB, AC, SR

Overall responsibility: SG

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**Competing Interests Statement**: NO authors have competing interests

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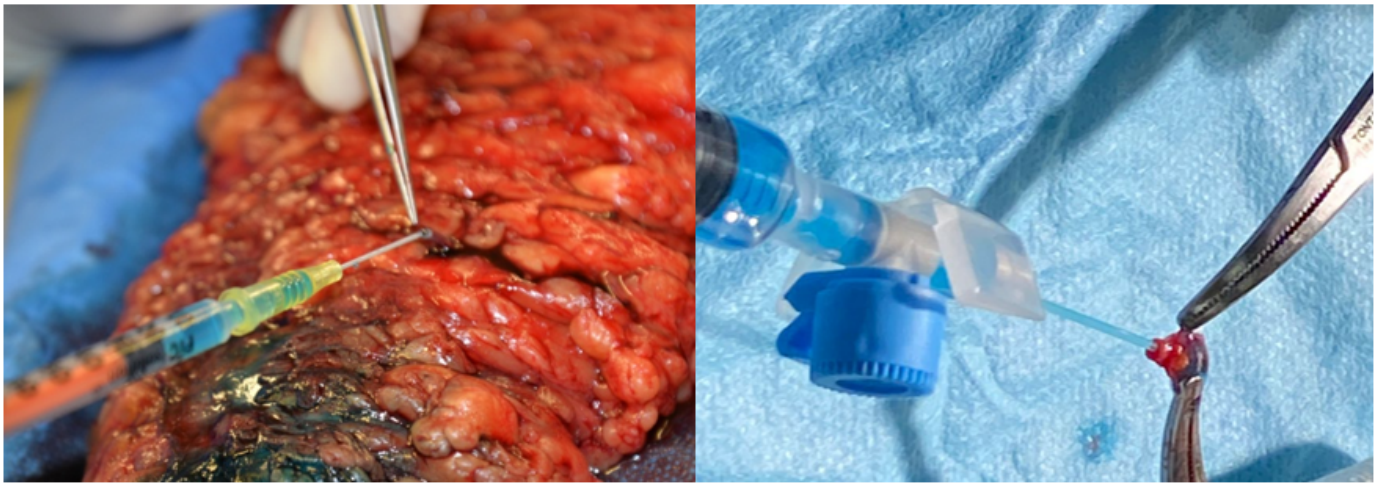
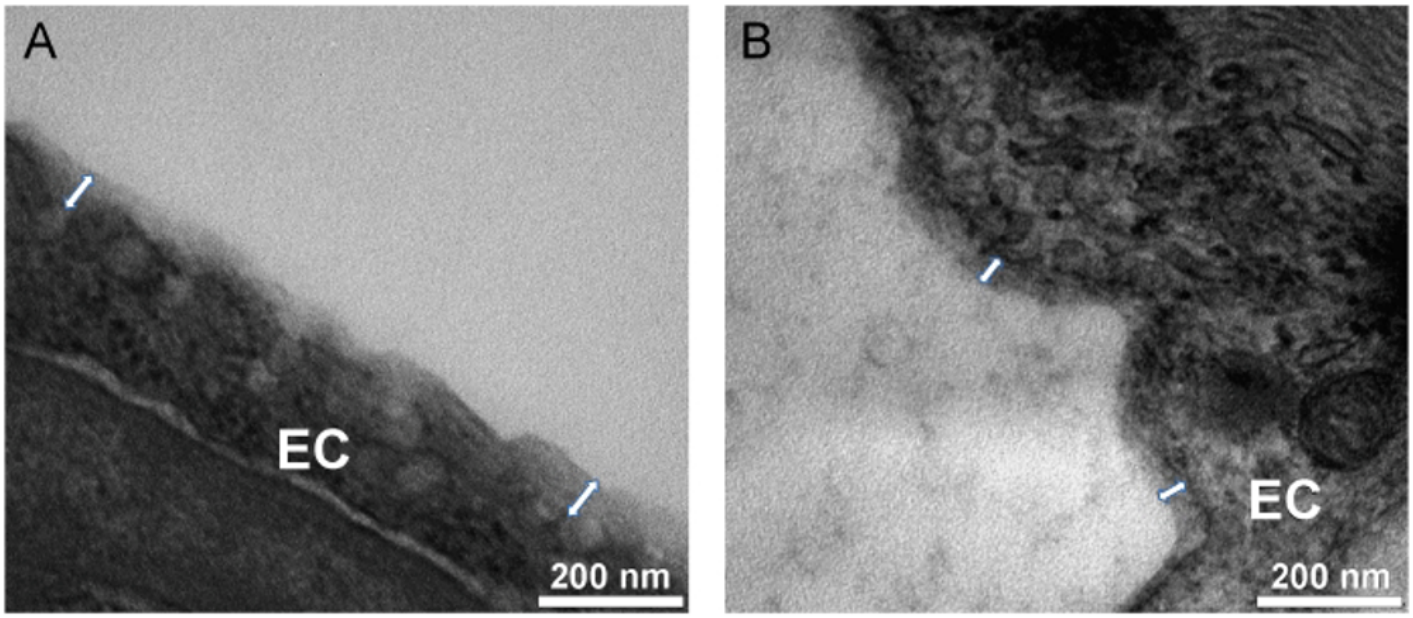
# Figures



A B

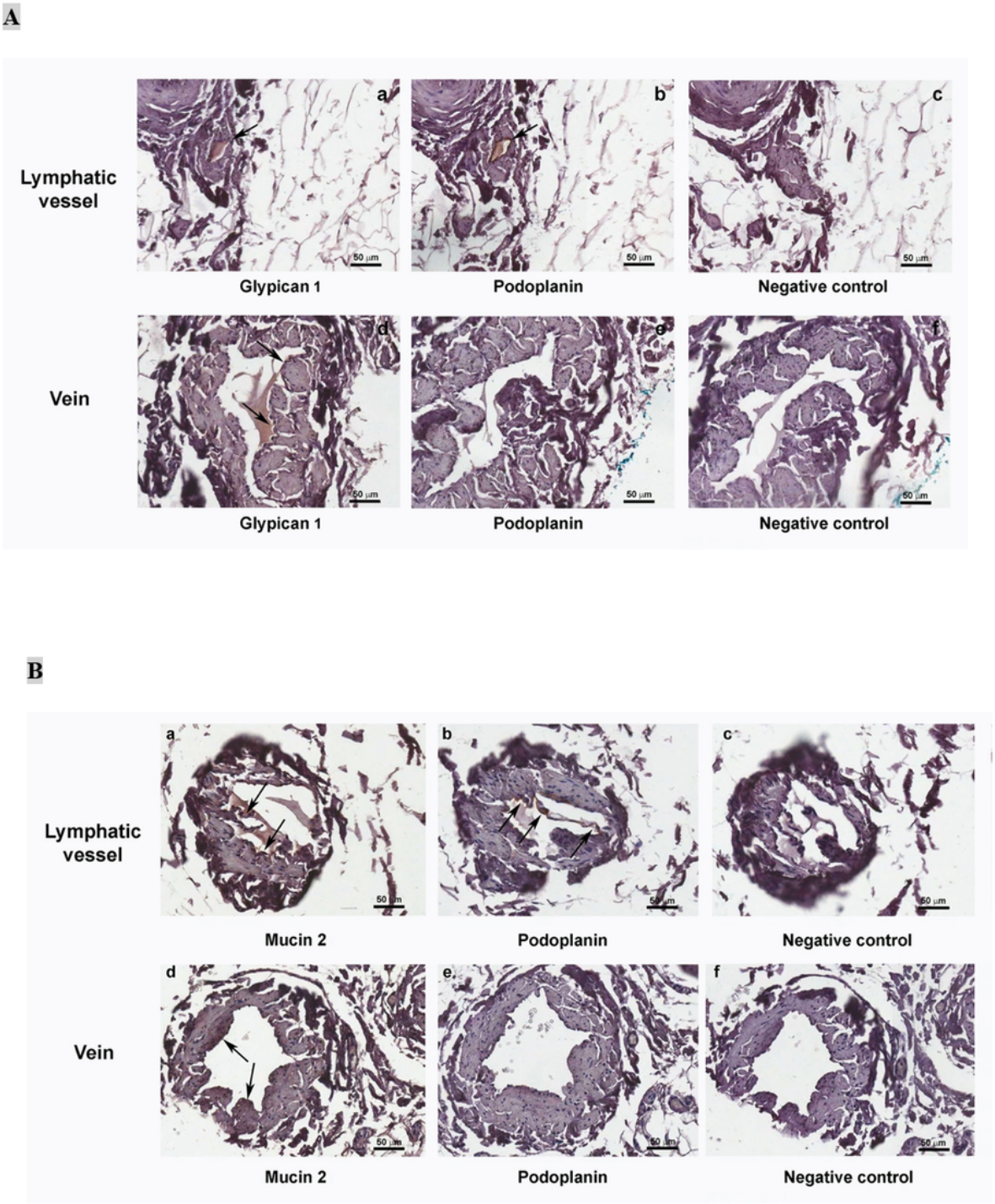
**Figure 1**

Fixative flush in the lymphatic vessel (A) and in the vein (B)



## Figure 2

**Glycocalyx assessment of lymphatic vessels.** High-magnification transmission electron micrographs (scale bar: 200 nm) showing the endothelial glycocalyx in a vein (A) and in a lymphatic vessel (B) fixed with glutaraldehyde in the presence of Alcian Blue 8GX. EC indicates endothelial cells. Double white arrow indicates the glycocalyx layer.




## Figure 3

**Immunohistochemistry showing lymphatic glycocalyx components.** Immunohistochemical analyses were performed in various sections of lymphatic vessels and veins by means of antibodies to anti-podoplanin, anti-glypican 1 and anti-mucin-2 (scale bar: 50 mm). Black arrows were added to allow a better identification of the specific labeling.