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Graphical abstract

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Platelet factor 4 is a biomarker for lymphatic-promoted disorders

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The authors have declared that no conflict of interest exists.

**Abstract:**

Genetic or acquired defects of the lymphatic vasculature often result in disfiguring, disabling and, occasionally, life-threatening clinical consequences. Advanced forms of lymphedema are readily diagnosed clinically, but more subtle presentations often require invasive imaging or other technologies for a conclusive diagnosis. On the other hand, lipedema, a chronic lymphatic microvascular disease with pathological accumulation of subcutaneous adipose tissue is often misdiagnosed as obesity or lymphedema; currently there are no biomarkers or imaging criteria available for a conclusive diagnosis. Recent evidence suggests that otherwise asymptomatic defective lymphatic vasculature likely contributes to an array of other pathologies, including obesity, inflammatory bowel disease and neurological disorders, among others. Accordingly, identification of biomarkers of lymphatic malfunction will provide a valuable resource for the diagnosis and clinical discrimination of lymphedema, lipedema, obesity and other potential lymphatic-related pathologies. In this paper we profiled and compared blood plasma exosomes isolated from mouse models and from human subjects with and without symptomatic lymphatic pathologies. We identified platelet factor 4 (PF4/CXCL4) as a biomarker that could be used to diagnose lymphatic vasculature dysfunction. Furthermore, we determined that PF4 levels in circulating blood plasma exosomes were also elevated in lipedema patients, supporting current
claims arguing that at least some of the underlying attributes of this disease are also the consequence of lymphatic defects.

**Introduction**

The lymphatic vasculature is a network of thin-walled initial lymphatic capillaries and larger collecting vessels covered by a continuous layer of endothelial cells providing a unidirectional conduit for filtered tissue interstitial fluids, metabolites, macromolecules and cells toward the central venous circulation. Its principal function is to maintain fluid homeostasis by removing the protein-enriched fluids from the extracellular space and returning them, in the form of lymph, to the bloodstream (1). Lymphatics are also important for lipid transport and immune cell trafficking, among other functions.

One of the main disorders that ensue from malfunction of the lymphatic vasculature is lymphedema, a disfiguring, disabling, and occasionally, life-threatening clinical condition characterized by the localized interstitial accumulation of protein-rich fluid, thereby promoting tissue edema for which, at present, treatment options are few and efficacy is limited (2). This disease affects millions of persons worldwide, and most commonly entails swelling of the extremities, tissue fibrosis, susceptibility to infections and accumulation of subcutaneous fat (2,3). Lymphedema can result from either primary or acquired (secondary) disorders. Primary lymphedema is the consequence of genetic defects that impact the formation and normal function of the lymphatic vasculature, and most commonly manifests during infancy, childhood or adolescence (2,4). Secondary lymphedema is the more common presentation and is caused by lymphatic trauma sustained after surgery, radiation therapy, infection or trauma (2–4). In general, overt lymphedema can be diagnosed based on the clinical context and the physical examination;
however, more precise staging and characterization requires imaging protocols that are often invasive.

A direct correlation and mechanistic relationship between the lymphatic vasculature and the adipose compartment have recently been recognized in patients with lymphatic disorders. Abnormal subcutaneous fat accumulation in the affected edematous regions in patients with secondary lymphedema is the inescapable consequence of sustained defective lymphatic drainage. Analysis of patients has also shown that malformation of cutaneous lymphatics causes bilateral fat accumulation in the thigh and buttock (5–7), a phenotype that worsens during puberty, while dermal lipid accumulation occurs in idiopathic lymphedema patients (8,9). Although physiotherapy and use of compression garments do limit interstitial fluid accumulation, at present there are limited options for the treatment of these more advanced manifestations of the disease.

Lipedema is a common, chronic lymphovascular disease (10–13) characterized by bilateral, symmetrical swelling in the extremities due to the deposition of abnormal subcutaneous adipose tissue (10,11,14). Lipedema, often misdiagnosed as obesity or lymphedema (10,11,15–17), occurs almost exclusively in females and has a likely genetic component, as a positive family history is common. Nevertheless, in contrast to lymphedema, overt interstitial edema is not observed in lipedema, and the swelling due to adipose hypertrophy occurs in a distinctly symmetrical pattern (16). Early studies by Bilancini et al., demonstrated that lipedema is consistently associated with functional alterations of the lymphatic vasculature (11). Using dynamic imaging, they showed that patients suffering from lipedema have an abnormal lymphoscintigraphic pattern, with a slowing of the lymphatic flow similar to the alterations found in lymphedema patients (11). Despite these insights, lipedema is frequently misdiagnosed as obesity or lymphedema, and the pathogenesis and molecular mechanisms of this disease are still very poorly understood.
Nevertheless, lipedema appears to be an adipose disorder with an apparent contribution of lymphatic malfunction. Whether those lymphatic alterations are partially responsible for the disease, or are secondary to the related obesity features is not yet known. Unfortunately, even with focused morphological analysis, lipedema is not easy to differentiate from obesity; clinicians often lack familiarity with this condition, distinct clinical imaging attributes have not been identified, there are no known biomarkers for the disease, and conclusive mechanistic evidence supporting the proposal that lymphatic defects contribute to the disease is still lacking.

More recently, the functional roles of the lymphatic vasculature have broadened. New evidences suggest that asymptomatic defective and/or leaky lymphatic vessels could be responsible for certain forms of obesity (18,19), inflammatory bowel disease/Crohn’s disease, glaucoma and some forms of neurological pathology [for a review see (20)]. Thus, identification of easily accessible, reliable biomarkers of lymphatic malfunction would be a valuable resource to assist not only in the conclusive diagnosis of lymphedema, but also to facilitate the differential diagnosis among lymphedema, lipedema and obesity subjects. Furthermore, the identification of such biomarkers could eventually also help to identify and diagnose subtle, asymptomatic lymphatic alterations that might contribute to some of the aforementioned disorders. Accordingly, we profiled and compared circulating exosomes isolated from blood plasma from animal models and from patients with and without documented lymphatic pathologies. Exosomes are small vesicles (30-100 nm in diameter) of endocytic origin secreted by most cells (including endothelial cells)(21–24). These extracellular vesicles contain cell type-specific proteins and genetic materials, including mRNAs, miRNAs and DNA. They can also exert a functional influence once taken up by recipient cells, therefore representing novel mediators of intercellular communication(25–30). Exosomes are emerging biomarkers of various types of diseases (21).
In this investigation, we performed mass spectrometry (MS) analysis and compared exosome proteomic signatures in normal, obese and lymphatic defective mouse models. A similar approach was used with plasma exosomes obtained from patients with various lymphatic disorders, with lipedema, and from obese and non-obese individuals without clinically overt lymphatic dysfunction. We report on the identification of platelet factor 4 (PF4) as a plasma-circulating exosomal signature protein that could be used as a potential novel biomarker in the clinical setting to diagnose lymphatic vasculature dysfunction, and to distinguish these disorders from non-lymphatic-promoted obesity. Furthermore, we also found that PF4 levels were also increased in circulating exosomes from lipedema patients, a result that supports the prevailing hypothesis that the pathogenesis of this disease is, at least in part, lymphatic. However, exosomal PF4 levels are not associated with increased body weight, either in individuals with normal lymphatics or those with lymphatic-associated disorders.

Results

Exosomes profiling in a mouse model of lymphatic malfunction

In an initial approach to determine whether blood plasma-derived exosomes might be used to identify lymphatic vascular defects, we first used available mouse models. We have previously reported that haploinsufficiency of Proxl in mice results in morphological and functional alterations in the lymphatic vasculature that is associated with edema at mid-gestation and with obesity in adult animals(18). Detailed characterization of the lymphatic vasculature of E14.5 Proxl+/− embryos showed that they displayed edema, indicating lymphatic dysfunction, but this phenotype resolved before birth (18). Detailed characterization of the lymphatic vasculature of E16.5 Proxl+/− embryos and adult Proxl+/− mice revealed mispatterning of the lymphatic vasculature; the most severely affected lymphatics were those of the intestine and mesentery,
which were chyle-filled and ruptured (18,19). A low percentage of Prox1\(^{+/−}\) mice survive to adulthood and become significantly heavier than WT littermates at approximately 4 months of age, a consequence of the subtle leakage of lymph/chyle that promotes visceral accumulation of fat leading to obesity (18,19). Accordingly, we compared the protein profile of plasma-circulating exosomes from young non-obese (<3 months) and older obese (> 5 months) Prox1\(^{+/−}\) mice, WT littermates and Ob/Ob mice (leptin receptor mutants) (31–33) that are severely obese but have a normal lymphatic vasculature (our unpublished results) (for each model we used mice of both sexes). We reasoned that by comparing those groups we should be able to identify biomarkers capable of distinguishing lymphatic malfunction (Prox1\(^{+/−}\) mice) from non-lymphatic-promoted obesity (i.e., Ob/Ob mice) and from WT mice.

To isolate exosomes terminal bleeding was performed, and blood was collected by cardiac puncture. Circulating exosomes were purified from the isolated plasma using standard protocols (see Materials and Methods for more information), and their presence and particle size was confirmed by Nanosight (34) and by electron microscopy (data not shown). Consistently, we found that in Prox1\(^{+/−}\) mice, the number of exosomes was higher than their age-matched littermate controls, either before or after the onset of obesity (Fig. 1). Next, exosomes were subjected to mass spectrometry (MS) to identify their protein cargo components. Due to the low survival rate of Prox1\(^{+/−}\) mice and the low plasma volume, the exosome yield was low. Therefore, for the MS analysis, plasma samples of animals with the same genotype were pooled. We initially compared the proteomic signature of young (lean 3 month-old) and old (obese 5 month-old) Prox1\(^{+/−}\) mice and age-matched WT littermates using a fold change cutoff of >0.5 or <0.5 to identify the early changes that persists as disease develops. Using those criteria, 70 proteins were up-regulated in both young and old Prox1\(^{+/−}\) mice (Fig. 2A) and 36 were down-regulated (Fig. 2B). Important for
the findings described below using the human samples, among the upregulated ones it was platelet factor 4 (PF4, Fig. 2A). Pathway enrichment and protein-protein interaction network analysis (Encyclopedia of Genes and Genomes, KEGG) revealed that the upregulated proteins were mainly enriched in complement and coagulation cascades and systemic lupus erythematosus pathways (Table 1); in contrast, the downregulated ones were mainly identified in proteasome, Epstein-Barr virus infection and leukocyte trans-endothelial migration pathways (Table 2).

We then performed a similar MS analysis using pooled plasma from Ob/Ob and WT mice. Among the 479 proteins, 187 were increased and 75 were decreased in the Ob/Ob group (Fig. 3A). To exclude proteins related to obesity, we then compared the Prox1+/− mice dataset with the ones from WT and Ob/Ob mice. We identified 9 upregulated proteins and 2 downregulated proteins common to Prox1+/− and Ob/Ob mice, and narrowed the lymphatic specific signature in Prox1+/− to 61 upregulated and 34 downregulated proteins (Fig. 3B-C).

**Isolation and characterization of exosomes from lymphedema patients**

To further validate and expand the animal model results described above, we next performed a similar analysis with plasma-circulating exosomes isolated from patients with lymphatic dysfunction and from normal subjects. To do this we performed an initial pilot experiment; although the pilot study included a relatively limited number of subjects, the patient cohorts were generally well-matched by demographic variables (Table 3). The studied cohorts included lean and obese healthy subjects without overt lymphatic dysfunction, and patients with lymphatic disorders, including lean and obese subjects with secondary lymphedema, lean and obese subjects with lymphovascular disease, and lean and obese subjects with lipedema. As expected, all of the lymphatic disease cohorts were female-predominated (Table 3). Also, as anticipated, the category
of lymphovascular disease, which reflects developmental and genetic diseases, is characterized by a significantly younger mean age.

Initially, we focused on the molecular differences between normal individuals and lymphatic patients, and therefore did not segregate individuals by BMI. Following exosome purification and MS analysis, we profiled 4 samples pooled from 8 normal subjects without overt lymphatic dysfunction, 8 pooled samples from 15 secondary lymphedema patients, 3 samples from 3 lymphovascular patients and 8 samples from 8 lipedema patients. Proteins with a p.value < 0.1 and a log2 ratio >1 or <-1 were considered to be differentially regulated. From this analysis, we identified 13 increased and 14 decreased proteins in secondary lymphedema patients (Table 4-5), 38 increased and 55 decreased proteins in lymphovascular disease patients (Table 6-7, only the top 50 decreased proteins are shown due to space limits) and 19 increased and 35 decreased proteins in lipedema patients (Table 8-9). Of interest, and as shown in Tables 4, 6 and 8, among this list of upregulated proteins, platelet factor 4 (PF4) was the only one whose levels were elevated (when compared with normal controls) in samples from secondary lymphedema, lymphovascular disease (including primary lymphedema), lipedema patients and also in Prox1+/− mice (Fig. 2A). Platelet factor 4 is a protein released from platelets and is known to be able to inhibit angiogenesis and to promote innate immune responses, making this protein an interesting target for inflammation. PF4 bound to surface glycosaminoglycans on platelets, monocytes and endothelial cells is also an immunogenic target in prothrombotic disorders. The concentration of PF4 in serum after platelet activation is a thousand-fold higher than in plasma (35–38).

Next, we decided to further validate these initial results using a human PF4 ELISA assay. The exosomes protein cargo from 12 normal subjects, 37 lymphedema patients, 11 lymphovascular disease patients and 15 lipedema patients was analyzed (protein content was normalized for each
This ELISA analysis validated the MS results described above, as PF4 levels were elevated in all patients except one (Fig. 4A); as determined by Grubb’s test (39), this single non-lymphedema obese patient with very high PF4 levels was an outlier with a history of inflammatory bowel disease; therefore, it was removed from this graph (Fig. 4A). Although the pathophysiology of IBD remains unknown, alterations in the intestinal lymphatics are becoming accepted features of IBD, particularly in Crohn’s disease subjects (40–42). To evaluate the diagnostic power of PF4 for lymphatic alterations, a receiver operating characteristic (ROC) curve analysis was performed. As shown in Fig. 4B, the areas under the ROC Curve (AUCs) were 0.80 (95% confidence interval (CI) 0.67 to 0.93), 0.86 (95% CI: 0.70 to 1.00) and 0.95 (95% CI: 0.99 to 1.00) for secondary lymphedema, lymphovascular disease and lipedema patients, respectively. At the corresponding optimal cut-off values, the sensitivities and specificities of PF4 to predict secondary lymphedema reached 59.46% and 90.91%, for lymphovascular reached 70.00 % and for lipedema reached 86.67% and 90.91%, respectively.

To eliminate the likelihood that co-morbidities might be responsible for the observed differences in PF4 levels, we examined the distribution of co-morbidities among the subjects in each of the enrolled cohorts (Table 10). The only significant differences observed were that of a reduced incidence of cancer in the lipedema cohort when compared to those with lymphedema, and increased hypertension and musculoskeletal disease in the control group when compared to those with lymphedema. Of note, no identified platelet disorder was listed among these patients. In parallel with the human clinical observations, PF4 was also upregulated in both young and old Prox1+/− mice (Fig. 2B) but not in Ob/Ob mice. These findings suggest that PF4 could be a novel biomarker for lymphatic disorders.
Then, to explore if PF4 can distinguish normal lean and obese human subjects (not symptomatic lymphatic malfunction), from those with lymphatic disorders, we further separated lean and obese normal and lymphatic-affected individuals. As shown in Figure 4C-D, the PF4 level is not statistically different in lean or obese normal or affected subjects. This suggests that PF4 is a promising biomarker capable to distinguish normal subjects from those with lymphatic defects independent of the presence or absence of obesity.

**Discussion**

Lymphedema is a devastating disease that lacks early diagnostic tools and readily available pharmacological interventions. Current accurate diagnosis of early or subclinical disease often relies upon sophisticated imaging techniques, which can be relatively invasive. Less invasive screening tools are not yet available. Although the more advanced stages of lymphedema can be clinically diagnosed, subtle, early, and subclinical disease can be elusive. Furthermore, with the recent surge of newly identified functional roles for the lymphatic vasculature in a variety of normal and pathological conditions (*e.g.*, obesity, cardiovascular disease and neurodegenerative disorders), it is possible that individuals with any of those pathologies might be grossly asymptomatic for any of the typical features of lymphatic dysfunction. In such circumstances, the ready availability of reliable biomarkers could play a defining role in the screening and diagnosis of more subtle forms of lymphatic-associated defects.

This analysis, using mouse models and individuals with a variety of lymphatic pathologies, identified PF4 as a promising diagnostic marker for lymphatic-promoted disorders. The levels of PF4 were increased in both young and old *Prox1*<sup>+/−</sup> mice (before and after the onset of obesity), as well as in lymphedema, lipedema and patients with heritable developmental diseases of the
lymphatics. PF4, also called CXCL4, is a chemokine that is packaged in platelet alpha-granules and is secreted upon activation during inflammation and wound healing. Although this study does not identify the cell of origin of exosomal PF4 or the mechanism underlying exosomal PF4 secretion, it is possible to speculate that structurally and functionally defective lymphatics are responsible for mediating such signaling. Besides regulating hemostasis and thrombosis, platelets also play an important developmental role in the separation of the blood and lymphatic vascular networks (43,44). Platelets are activated by lymphatic endothelial cells to form a plug at the level of the lymphovenous valve, the structure where the central lymphatic vasculature connects to the blood vascular system. In mice, the failure to form such a platelet plug results in the reflux of blood into the lymphatic vessels and these mice develop lymphedema (45,46). Interestingly, it has been reported that PF4 is also increased in a mouse model of acute surgical lymphedema detected by cDNA microarray analysis (47). Prior studies suggested that PF4 inhibits angiogenesis in vivo and in vitro (48,49). For example, PF4 inhibits FGF2 and VEGF signaling through heparin-dependent and -independent mechanisms (50,51). However, whether increased exosomal PF4 inhibit lymphangiogenesis in vivo is not clear.

A few lines of evidence support the hypothesis that PF4 might play a role in lymphedema. It has been shown that PF4 induces chemotaxis of T lymphocytes and upregulates T helper 2 (Th2) cytokines in a CXCR3 dependent manner (52,53). This is relevant because T cells, including Th2 cells, are known to infiltrate lymphedematous tissue and play a role in inflammation, fibrosis and lymphangiogenesis (54–56). Blocking Th2 differentiation decreases fibrosis, improves lymphatic function and delays the progression of lymphedema (56). The elevated levels of PF4 detected in plasma-circulating exosomes of affected individuals might contribute to the recruitment and stimulation of Th2 cells in lymphedema patients. Moreover, it has been also reported that blood
plasma PF4 levels are increased in patients with Crohn's disease (57–63), a disorder that has been recently shown to feature lymphatic alterations (42,64). The elevated platelet count appears to correlate with the presence of immature platelets in blood, that might play a role in predisposing IBD patients to thrombus development. It is reasonable to speculate that the increased levels of PF4 in Crohn’s disease could also, at least partially, be the consequence of the associated alterations in the mesenteric lymphatic vasculature. In addition, lipopolysaccharides (LPS), which trigger pro-inflammatory responses in endothelial cells, increase PF4 levels and cell permeability by reducing tight junction proteins in cultured human umbilical vein endothelial cells (HUVEC) (65). The authors suggested that the effect of LPS on cell permeability is mediated by PF4, since it can be abolished by PF4 neutralizing antibodies, and PF4 itself decreases tight junction proteins and promotes cell permeability (65). Potentially, it could be speculated that PF4 might increase blood vessel permeability and reduce lymphangiogenesis as contributing factors in lymphatic diseases. Supporting this hypothesis, pathological alterations of leukotriene biology have been observed in both murine and human lymphedema, with evidence of anti-lymphangiogenic concentrations of leukotriene B4 (LTB4) in these individuals (66). It is notable that LTB4 also induced endothelial cell permeability in vivo (67). Future investigations of the role of PF4 in lymphatic dysfunction should encompass exploration of the relationship of PF4 to leukotriene-mediated effects in the pathogenesis of lymphedema and lymphatic-related disorders.

Several studies have suggested a close association of excessive fat accumulation with lymphatic dysfunction. We have previously shown that Prox1+/− mice with defective lymphatics develop adult-onset obesity, likely a consequence of chyle leakage (18,68). Comparison of the exosome protein profile between Prox1+/− and Ob/Ob mice demonstrated substantial differences and, specifically, PF4 was not increased in Ob/Ob mice. These data suggest that PF4 levels might also
be useful to identify obese individuals in which at least some of the underlying pathogenesis of excessive fat accumulation could be subtle and asymptomatic lymphatic leakage. Finally, our results support the prevailing hypothesis that in lipedema, lymphatic dysfunction plays a role in the pathogenesis of the disease, as has previously been suggested on the basis of imaging attributes (10–13).

Some questions to be addressed in future studies include: a) as PF4 RNA levels are increased in the subacute tail wound model of acquired lymphedema (47), additional studies are needed to determine whether PF4 is increased in local tissue or circulating exosomes; b) in vivo lymphedema models using PF4−/− mice or anti-PF4 antibodies combined with platelet transfusion should provide insight about potential therapeutic application of PF4; (c) appropriately powered future clinical investigations of lymphatic disease cohorts are necessary to explore the relationship of PF4 levels with disease mechanisms and disease severity.

Materials and Methods

Mouse studies. Ob/Ob mice were obtained from the Jackson Laboratory (69). Prox1+/− mice were generated and reported previously (70).

Exosomes purification and characterization

Gently mixed blood with EDTA was centrifuged at 500xg for 10 min at 10 °C. Supernatant was centrifuged at 3000xg for 20 min at 10 °C. Plasma was centrifuged at 12,000xg for 20 min at 10 °C to remove microvesicles and the supernatant was centrifuged at 100,000xg for 70 min at 10 °C. The exosomes in the pellet fraction were washed with 20ml of PBS and centrifuged at 100,000xg for 70 min at 10 °C. The final exosome pellet was resuspended in 100 µl of PBS for analysis.
**Human studies**

We recruited study subjects from the patient population of the Stanford Center for Lymphatic and Venous Disorders. The Administrative Panels for the Protection of Human Subjects of Stanford University (IRB 0000350) approved the protocols. Investigations were conducted according to the Declaration of Helsinki principles. Written consent was obtained from all recipients prior to inclusion in the studies. Phlebotomy was performed in the standard fashion, using a small gauge needle inserted into the brachiocephalic vein. 30 cc of blood were withdrawn in EDTA tubes, and the plasma was frozen at −80°C for subsequent molecular analysis.

In order to be eligible for enrollment in this study, subjects were screened for the presence of lymphedema (primary or secondary), lipedema and lymphatic malformations. The diagnosis of lymphedema was based upon clinical evaluation, utilizing the criteria established by the International Society of Lymphology. The diagnosis of lipedema is based on commonly accepted clinical attributes (71). Normal control subjects were recruited from the same cardiovascular clinic as those with lymphatic pathologies; eligibility for enrollment included the absence of any clinically identifiable lymphatic pathology and the willingness to participate. In each subject cohort, the presence of obesity was defined as a BMI > 30.

**Mouse proteomic analysis**

Proteins were dissolved using 8 M urea in 100 mM ammonium bicarbonate and 10 mM DTT. After reduction, cysteines were alkylated in 30 mM iodoacetamide. Proteins were then in-solution and digested with Lys-C (endoproteinase LysC, Wako Chemicals) in 4 M urea, followed by
trypsinization (Trypsin Gold, Promega) in 2 M urea. Digestions were stopped by adding TFA and the digestes were desalted using C18 stage-tips.

Samples were analyzed by LC–MS/MS (Dionex 3000 coupled to Q-Exactive, Thermo Fisher). Peptides were separated by C-18 chromatography (inner diameter of 75 µm/3 µm particles, Nikkyo Technologies) using a gradient increasing from 1% B to 45% B in 135 min (A: 0.1% formic acid, B: acetonitrile in 0.1% formic acid). The peptides were electrosprayed (3.4 kV) into the mass spectrometer through a heated capillary at 320 °C and a S-Lens RF level of 60%. The mass spectrometer was operated in a data-dependent mode, with an automatic switch between the MS and MS/MS scans using a top 20 method (minimum AGC target 3E3) and a dynamic exclusion time of 45 sec. MS (300-1400 m/z) and MS/MS spectra were acquired with a resolution of 70,000 and 17,500 FWHM (200 m/z), respectively. Peptides were isolated using a 2 Th window and fragmented using higher-energy collisional dissociation (HCD) at 27% normalized collision energy. The ion target values were 5E5 for MS (100 ms maximum injection time) and 2E5 for MS/MS (60 ms maximum injection time).

Raw files were processed with MaxQuant (v 1.5.1.2) using the standard settings against a mouse protein database (UniProtKB/Swiss-Prot/TrEMBL, 43,539 sequences) supplemented with contaminants. Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionine and protein N-term acetylation as variable modifications. Minimal peptide length was set to 7 amino acids and a maximum of two tryptic missed cleavages were allowed. Results were filtered at 0.01 FDR (peptide and protein level). An arbitrary criteria of fold-change > 0.5 or <-0.5 was used to define proteins as up or down regulated.

**Human proteomic analysis**
Proteins were dissolved using 8 M urea in 100 mM Tris-HCl pH 8.0. Protein concentration was determined using the Pierce® 660nm Protein Assay (Bio-Rad) using BSA as standard. Then, samples (10-20 µg) were digested by means of the standard FASP protocol. Briefly, proteins were reduced and alkylated (15 mM TCEP, 30 mM CAA, 30 min in the dark, room temperature) and sequentially digested with Lys-C (Wako) (protein:enzyme ratio 1:50, o/n at RT) and trypsin (Promega) (protein:enzyme ratio 1:100, 6 h at 37 °C). Resulting peptides were desalted using C18 stage-tips.

LC-MS/MS was done by coupling a nanoLC-Ultra 1D+ system (Eksigent) to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) via a Nanospray Flex source (Thermo Fisher Scientific). Peptides were loaded into a trap column (NS-MP-10 BioSphere C18 5 µm, 20 mm length, Nanoseparations) for 10 min at a flow rate of 2.5 µl/min in 0.1% FA. Then peptides were transferred to an analytical column (ReproSil Pur C18-AQ 1.9 µm, 400 mm length and 0.075 mm ID) and separated using a 150 min linear gradient (buffer A: 4% ACN, 0.1% FA; buffer B: 100% ACN, 0.1% FA) at a flow rate of 250 nL/min. The gradient used was: 0-2 min 2% B, 3-133 min 30%B, 134-144 min 98% B, 145-150 min 2% B. The peptides were electrosprayed (1.8 kV) into the mass spectrometer with a PicoTip emitter (360/20 Tube OD/ID µm, tip ID 10 µm) (New Objective), a heated capillary temperature of 325° C and S-Lens RF level of 60%. The mass spectrometer was operated in a data-dependent mode, with an automatic switch between MS and MS/MS scans using a top 20 method (threshold signal ≥ 800 counts and dynamic exclusion of 45 sec). MS spectra (350-1500 m/z) were acquired in the Orbitrap with a resolution of 60,000 FWHM (400 m/z). Peptides were isolated using a 1.5 Th window and fragmented using collision induced dissociation (CID) with linear ion trap read out at a NCE of 35% (0.25 Q-value and 10 ms
activation time). The ion target values were 1E6 for MS (500 ms max injection time) and 5000 for MS/MS (100 ms max injection time).

Raw files were processed with MaxQuant (v 1.5.3.30) using the standard settings against a human protein database (UniProtKB/Swiss-Prot, December 2013, 20,584 sequences) supplemented with contaminants. Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionine and protein N-term acetylation as variable modifications. Minimal peptide length was set to 7 amino acids and a maximum of two tryptic missed cleavages were allowed. Results were filtered at 0.01 FDR (peptide and protein level). Afterwards, the “proteinGroups.txt” file was loaded in Prostar (v1.18) (72) using the intensity values for further statistical analysis. Briefly, proteins with less than 4 valid values in at least one experimental condition were filtered out. Then, a global normalization of log2-transformed intensities across samples was performed using the LOESS function. Missing values were imputed using the algorithms SLSA (73) for partially observed values and DetQuantile for values missing on an entire condition. Differential analysis was done using the empirical Bayes statistics Limma. Proteins with a p.value < 0.1 and a log2 ratio >1 or <-1 were defined as regulated. The FDR was estimated to be up to 10% by Benjamini-Hochberg

ELISA

Total protein quantification in exosomes was performed using the BCA protein assay kit (Pierce). PF4 concentration in exosomes was quantified using a human PF4 ELISA kit (R&D) according to the manufacturer’s instructions. The standards and the samples were run in duplicates. The results were read using a Synergy 2 plate reader. PF4 concentration was normalized with total protein content in exosomes.
Statistical Analysis

The analysis method for MS, demographic and co-morbidities was detailed in the text and table legends. All statistical analyses for ELISA were performed using GraphPad Prism 8.0. Data with parametric distribution were analyzed using unpaired Student t tests or 1-way analysis of variance (ANOVA); data with nonparametric distribution were analyzed by the Kruskal–Wallis test unless specified in the legends. All analyses with $P$ values below 0.05 were considered statistically significant. Data represent mean value ± standard error of the mean (s.e.m.).

Study approval

All the mouse work was approved by the Northwestern University Animal Care and IACUC guidelines. Study subjects were recruited from the patient population of the Stanford Center for Lymphatic and Venous Disorders. The Administrative Panels for the Protection of Human Subjects of Stanford University (IRB 0000350) approved the protocols. Investigations were conducted according to the Declaration of Helsinki principles. Written consent was obtained from all recipients prior to inclusion in the studies.

Author contributions: W.M. and G.O. designed the experiments. W.M. conducted the experiments, acquired and analyzed the data. H.J., N.E. conducted experiments and acquired data. S.R. provided patients samples and performed the demographic and co-morbidity analysis. P.X.E, A.B.M., J.M. conducted MS experiments and analysis. W.M, H.P., S.R. and G.O. wrote the manuscript. All other authors declare that they have no competing interests.
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Fig. 1. Characterization of plasma exosomes from young and old Prox1+/− mice. Exosome particle concentration is compared between young and old WT and Prox1+/− mice (N=4-6). Data represent mean value ± standard error of the mean (s.e.m) and statistical analyses performed by unpaired t test. *P<0.05, ****P<0.0001.
**Fig. 2.** Protein signatures in plasma exosomes from young and old *Prox1*+/− mice. Proteins that are both increased (A) or decreased (B) in young and old *Prox1*+/− mice were compared to age-matched WT mice. Gene name in red highlights the common changes in *Ob/Ob* mice. (N=4-6)
**Figure 3**

(A) Pie chart shows up and downregulated protein changes in Ob/Ob mice compared to WT controls. (N=3) (B-C) Venn diagram shows the common and unique proteins in Prox1\(^{+/−}\) compared to Ob/Ob mice. The common proteins are presented in red fonts in Fig. 2A-B.
Fig. 4. Validation of PF4 levels in plasma exosomes from individuals with normal lymphatics and patients with lymphatic disorders. (A) ELISA quantification of PF4 levels in exosomes from control subjects and indicated groups of patients. PF4 levels were normalized to the exosome protein content. (red dots indicate outliers detected by Iterative Grubb’s test and are excluded from the statistical analysis. * indicates \( P \leq 0.05 \), ** indicates \( P \leq 0.01 \), *** indicates \( P \leq 0.001 \) compared to control.) (B) ROC curve of PF4 for each diagnosis. The cut-off value of PF4 with sensitivity and specificity, as well as AUC and CI are presented in the separate table below the figure. (AUC, area under the receiver operating characteristic curve; CI, confidence interval.) (C) The PF4 from individuals with normal lymphatics are further divided based on BMI >30, and the PF4 level from lean and obese individuals with normal lymphatics is not statistically different (red dot indicates the outlier in Figure 4A normal group detected by Iterative Grubb’s test group and is excluded from the statistical analysis). (D) The PF4 from individuals with secondary lymphedema, lymphovascular disease and lipedema are grouped into lean and obese based on BMI of 30, and the PF4 level from lean and obese individuals with lymphatic disorders is not statistically different (red dot indicates the outlier in Figure 4A lymphovascular disease group detected by Iterative Grubb’s test group and are excluded from the statistical analysis).
Tables

Table 1: KEGG pathways analysis of increased exosomal proteins in young and old $Prox1^{+/-}$ mice compared to age-matched WT mice

| path | KEGG Pathways | count in gene set | false discovery rate |
|------|---------------|------------------|----------------------|
| mmu04610 | Complement and coagulation cascades | 6 of 88 | 4.84E-05 |
| mmu05322 | Systemic lupus erythematosus | 4 of 92 | 0.0123 |
Table 2: KEGG pathway analysis of decreased exosomal proteins in young and old Prox1+/− mice compared to age-matched WT mice

| Pathway   | Description                  | Count in gene set | False discovery rate |
|-----------|------------------------------|-------------------|----------------------|
| mmu03050  | Proteasome                   | 8 of 45           | 4.13E-14             |
| mmu05169  | Epstein-Barr virus infection | 7 of 205          | 8.27E-08             |
| mmu04670  | Leukocyte transendothelial migration | 3 of 115 | 0.0051               |
Table 3: Demographics and disease characterization of the lymphatic subjects and normal controls

|                        | LYMPHEDEMA (N=37) | LIPEDEMA (N=15) | LYMPHOVASCULAR (N=11) | CONTROL (N=12) |
|------------------------|--------------------|-----------------|-----------------------|---------------|
| **Age (years)**        | 58 ± 13            | 61 ± 13         | 41 ± 17**             | 64 ± 14       |
| **Female Gender no. (%)** | 35 (95)           | 15 (100)        | 7 (64)*               | 5 (14)*       |
| **Post-menopausal (%)** | 7 (19)             | 3 (20)          | 3 (43)                | 1 (8)         |
| **White**              | 26 (70)            | 14 (93)         | 10 (91)               | 9 (75)        |
| **Black**              | 1 (3)              | 1 (7)           | 0                     | 1 (8)         |
| **Asian**              | 2 (5)              | 0               | 0                     | 1 (8)         |
| **Other**              | 8 (22)             | 0               | 1 (9)                 | 1 (9)         |
| **BMI**                | 34 ± 16            | 36 ± 9          | 33 ± 12               | 32 ± 7        |
| **Obesity no. (%)**    | 14 (38)            | 12 (80)*        | 6 (55)                | 7 (58)        |
| **Disease duration (years)** | 15 ± 12          | 32 ± 19 **       | 20 ± 7**              | -             |
| **Limbs Affected**     |                    |                 |                       |               |
| **Upper no. (%)**      | 8 (22)             | -               | 0                     | -             |
| **Lower no. (%)**      | 28 (76)            | 15 (100)        | 7 (100)               | -             |
| **Unilateral lower (%)** | 4 (11)             | -               | 2 (29)                |               |
| **Other (%)**          | 1 (3)+             | 1 (7)¶          | 0                     | -             |
| **Lymphedema ISL Stage** |                    |                 |                       |               |
| **Stage I**            | 1 (3)              | -               | -                     |               |
| **Stage II**           | 31 (84)            | -               | 6 (86)                | -             |
| **Stage III**          | 5 (13)             | -               | 1 (14)                | -             |
| **Lipedema Stage**     |                    |                 |                       |               |
| **Stage I**            | 4 (27)             | -               | -                     |               |
| **Stage II**           | 6 (40)             | -               | -                     |               |
| **Stage III**          | 5 (33)             | -               | -                     |               |
| **Stage IV**           |                    |                 |                       |               |
| **Lymphatic disease etiology** |                |                 |                       |               |
| **Primary lymphedema no. (%)** | -               | 7 (64)          | -                     |               |
| **Secondary lymphedema no. (%)** | 37 (100)         | -               | -                     |               |
| **Lipedema no. (%)**   | 15 (100)           | -               | -                     |               |
| **Lymphatic malformation (%)** | -               | 3 (27)          | -                     |               |
| **Lymphangiectasia (%)** | -               | 1 (9)           | -                     |               |
| **Cancer-related no. (%)** | 18 (49)           | -               | -                     |               |

Kruskal-Wallis tests were performed for all continuous variables, and statistical comparisons were performed relative to lymphedema, unless indicated. Continuous variables were subjected to Mann-Whitney testing and discontinuous variables to the Fisher Exact test. Lymphatic disease etiology was not statistically examined. Unless indicated, the subject populations did not differ statistically. * p<0.05, **p<0.01, ***p<0.001, compared to lymphedema subjects; + pelvic lymphedema only; ¶ 4-limb lipedema.
| **Protein names** | **Gene names** | **LogFC** |
|------------------|---------------|----------|
| Platelet factor 4; Platelet factor 4, short form | PF4 | 3.22 |
| Pregnancy zone protein | PZP | 1.75 |
| Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8 | IGHV4OR15-8 | 1.55 |
| Proto-oncogene tyrosine-protein kinase Src | SRC | 1.36 |
| Calcium-independent phospholipase A2-gamma | PNPLA8 | 1.32 |
| Tubulin alpha-4A chain | TUBA4A | 1.25 |
| Pleckstrin | PLEK | 1.18 |
| Ig kappa chain V-II region Cum | | 1.15 |
| Complement C2; Complement C2b fragment; Complement C2a fragment | C2 | 1.05 |
| Tubulin beta-1 chain | TUBB1 | 1.05 |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH | 1.02 |
| Serum amyloid A-4 protein | SAA4 | 1.02 |
| Haptoglobin; Haptoglobin alpha chain; Haptoglobin beta chain | HP | 1.01 |
Table 5: List of decreased proteins in secondary lymphedema patients compared to healthy individuals

| Protein names                                                                 | Gene names          | LogFC |
|-----------------------------------------------------------------------------|---------------------|-------|
| Reelin                                                                      | RELN                | -2.30 |
| Extracellular matrix protein 1                                              | ECM1                | -2.01 |
| von Willebrand factor;von Willebrand antigen 2                              | VWF                 | -1.84 |
| Tenascin                                                                    | TNC                 | -1.72 |
| Coagulation factor VIII;Factor VIIIa heavy chain, 200 kDa isoform;Factor VIIIa heavy chain, 92 kDa isoform;Factor VIII B chain;Factor VIIIa light chain | F8                  | -1.70 |
| Ig kappa chain V-I region AG                                                | TCP1                | -1.53 |
| T-complex protein 1 subunit alpha                                           | SLC4A1              | -1.47 |
| Band 3 anion transport protein                                              |                     | -1.27 |
| Ig heavy chain V-II region SESS                                              |                     | -1.27 |
| Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1 | SVEP1              | -1.25 |
| Moesin                                                                      | MSN                 | -1.25 |
| Ig alpha-2 chain C region                                                   | IGHA2               | -1.21 |
| Ig kappa chain V-I region AU                                                |                     | -1.17 |
| Catalase                                                                    | CAT                 | -1.16 |
Table 6: List of increased proteins in lymphovascular disease patients compared to healthy individuals

| Protein names | Gene names | LogFC |
|---------------|------------|-------|
| Ig kappa chain V-IV region; Ig kappa chain V-IV region JI | IGKV4-1 | 8.14 |
| Pregnancy zone protein | PZP | 3.46 |
| Ig kappa chain V-III region IARC/BL41 | | 3.03 |
| Ig lambda chain V-V region DEL | | 2.51 |
| Platelet factor 4; Platelet factor 4, short form | PF4 | 2.41 |
| C-reactive protein; C-reactive protein(1-205) | CRP | 2.27 |
| Ig kappa chain V-I region Mev | | 2.21 |
| Ig kappa chain V-III region NG9 | | 2.16 |
| Alcohol dehydrogenase class-3 | ADH5 | 1.99 |
| Peroxiredoxin-2 | PRDX2 | 1.86 |
| Desmoglein-1 | DSG1 | 1.81 |
| Ig kappa chain V-III region Ti | | 1.80 |
| Ig heavy chain V-III region TIL | | 1.79 |
| Ig lambda chain V-III region SH | | 1.77 |
| Ig heavy chain V-III region 23 | IGHV3-23 | 1.71 |
| Ig kappa chain V-II region MIL | | 1.56 |
| Complement C2; Complement C2b fragment; Complement C2a | C2 | 1.51 |
| Ig lambda chain V-VI region WLT; Ig lambda chain V-VI region EB4 | | 1.48 |
| Complement factor D | CFD | 1.45 |
| Proto-oncogene tyrosine-protein kinase Src | SRC | 1.34 |
| Ig lambda chain V-II region BUR | | 1.32 |
| Fibulin-5 | FBLN5 | 1.28 |
| Clathrin heavy chain 1 | CLTC | 1.24 |
| Ig kappa chain V-I region Wes | | 1.22 |
| Ig lambda chain V-I region WAH | | 1.21 |
| Ig lambda chain V region 4A | | 1.20 |
| Ig kappa chain V-II region TEW | | 1.20 |
| Fetuin-B | FETUB | 1.19 |
| Ig heavy chain V-I region V35 | | 1.14 |
| Complement factor H-related protein 1 | CFHR1 | 1.14 |
| T-complex protein 1 subunit gamma | CCT3 | 1.12 |
| Ig kappa chain V-I region BAN | | 1.11 |
| Complement C4-A; Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain | C4A | 1.11 |
| Junction plakoglobin | JUP | 1.11 |
| Ig lambda chain V-I region NIG-64; Ig lambda chain V-I region BL2 | | 1.11 |
| Ig lambda chain V-IV region MOL | | 1.10 |
| Ig lambda chain V-I region NEW | | 1.06 |
| Ig heavy chain V-III region HIL | | 1.02 |
Table 7: List of top 50 decreased proteins in lymphovascular disease patients compared to healthy individuals

| Protein names                                                                 | Gene names                                                                 | LogFC |
|------------------------------------------------------------------------------|----------------------------------------------------------------------------|-------|
| Coagulation factor VIII;Factor VIIIa heavy chain, 200 kDa isoform;Factor VIIIa heavy chain, 92 kDa isoform;Factor VIII B chain;Factor VIIIa light chain | F8                                           | -3.45 |
| Ficolin-3                                                                     | FCN3                                                                 | -3.43 |
| Integrin alpha-6;Integrin alpha-6 heavy chain;Integrin alpha-6 light chain;Processed integrin alpha-6 | ITGA6                                                     | -3.08 |
| Mannan-binding lectin serine protease 2;Mannan-binding lectin serine protease 2 A chain;Mannan-binding lectin serine protease 2 B chain | MASP2                           | -2.96 |
| Tubulin beta-1 chain                                                          | TUBB1                                                                 | -2.63 |
| Erythrocyte band 7 integral membrane protein                                 | STOM                                                                 | -2.47 |
| Tenascin-X                                                                    | TNXB                                                                 | -2.46 |
| Mannan-binding lectin serine protease 1;Mannan-binding lectin serine protease 1 heavy chain;Mannan-binding lectin serine protease 1 light chain | MASP1                           | -2.42 |
| Ig alpha-2 chain C region                                                     | IGH A2                                                                 | -2.37 |
| CD9 antigen                                                                   | CD9                                                                   | -2.37 |
| Actin, alpha skeletal muscle;Actin, alpha cardiac muscle 1;Actin, gamma-enteric smooth muscle;Actin, aortic smooth muscle | ACTA1;ACTC1;ACTG2;ACTA2 | -2.37 |
| Talin-1                                                                       | TLN1                                                                  | -2.35 |
| Filamin-A                                                                     | FLNA                                                                  | -2.33 |
| 14-3-3 protein zeta/delta                                                     | YWHAZ                                                                 | -1.99 |
| Band 3 anion transport protein                                                 | SLC4A1                                                                | -1.94 |
| Vinculin                                                                      | VCL                                                                   | -1.93 |
| Integrin alpha-IIb;Integrin alpha-IIb heavy chain;Integrin alpha-IIb light chain, form 1;Integrin alpha-IIb light chain, form 2 | ITGA2B                           | -1.90 |
| Tenascin                                                                      | TNC                                                                   | -1.88 |
| Apolipoprotein C-III                                                          | APOC3                                                                 | -1.85 |
| von Willebrand factor;von Willebrand antigen 2                                 | VWF                                                                   | -1.82 |
| Integrin beta-3                                                               | ITGB3                                                                 | -1.77 |
| Catalase                                                                      | CAT                                                                   | -1.73 |
| A disintegrin and metalloproteinase with thrombospondin motifs 13            | ADAMTS13                                                              | -1.68 |
| Tubulin alpha-4A chain                                                        | TUBA4A                                                                | -1.65 |
| Ubiquitin-60S ribosomal protein L40;Ubiquitin;60S ribosomal protein L40;Ubiquitin-40S ribosomal protein S27a;Ubiquitin;40S ribosomal protein S27a;Polyubiquitin-B;Ubiquitin;Polyubiquitin-C;Ubiquitin | UBA52;RPS27A;UBB;UBC | -1.65 |
| ADP-ribosylation factor 1;ADP-ribosylation factor 3;ADP-ribosylation factor 5;ADP-ribosylation factor 4 | ARF1;ARF3;ARF5;ARF4 | -1.63 |
| Gene Name | Short Name | log2 fold change |
|-----------|------------|-----------------|
| Apolipoprotein F | APOF | -1.62 |
| Hemoglobin subunit alpha | HBA1 | -1.62 |
| Integrin beta-1 | ITGB1 | -1.59 |
| Extracellular matrix protein 1 | ECM1 | -1.57 |
| Carbonic anhydrase 1 | CA1 | -1.57 |
| Actin, cytoplasmic 2; Actin, cytoplasmic 2, N-terminally processed | ACTG1 | -1.51 |
| Apolipoprotein B-100; Apolipoprotein B-48 | APOB | -1.49 |
| Fermitin family homolog 3 | FERMT3 | -1.46 |
| Apolipoprotein C-1; Truncated apolipoprotein C-1 | APOC1 | -1.45 |
| Ras suppressor protein 1 | RSU1 | -1.44 |
| Serum amyloid A-1 protein; Amyloid protein A; Serum amyloid protein A(2-104); Serum amyloid protein A(3-104); Serum amyloid protein A(2-103); Serum amyloid protein A(2-102); Serum amyloid protein A(4-101) | SAA1 | -1.43 |
| Hemoglobin subunit delta | HBD | -1.41 |
| Alpha-actinin-1 | ACTN1 | -1.41 |
| Moesin | MSN | -1.38 |
| Beta-parvin | PARVB | -1.37 |
| Adenyl cyclase-associated protein 1 | CAP1 | -1.35 |
| Basement membrane-specific heparan sulfate proteoglycan core protein; Endorepellin; LG3 peptide | HSPG2 | -1.33 |
| Hemoglobin subunit beta; LVV-hemorphin-7; Spinorphin | HBB | -1.28 |
| Coagulation factor XIII B chain | F13B | -1.27 |
| Ig heavy chain V-III region GA |  | -1.24 |
| Ficolin-2 | FCN2 | -1.24 |
| HLA class I histocompatibility antigen, A-68 alpha chain | HLA-A | -1.18 |
| Apolipoprotein C-II; Proapolipoprotein C-II | APOC2 | -1.18 |
| Alpha-2-HS-glycoprotein; Alpha-2-HS-glycoprotein chain A; Alpha-2-HS-glycoprotein chain B | AHSG | -1.13 |
Table 8: List of increased proteins in lipedema patients compared to healthy individuals

| Protein names                                                                 | Gene names                      | LogFC |
|-------------------------------------------------------------------------------|---------------------------------|-------|
| Ig kappa chain V-IV region; Ig kappa chain V-IV region JI                      | IGKV4-1                         | 7.29  |
| Platelet glycoprotein Ib beta chain                                           | GP1BB                           | 2.61  |
| Myosin-9                                                                      | MYH9                            | 2.32  |
| Pleckstrin                                                                    | PLEK                            | 2.13  |
| Myosin light polypeptide 6                                                    | MYL6                            | 2.12  |
| Ig kappa chain V-III region IARC/BL41                                         |                                 | 2.12  |
| Pregnancy zone protein                                                        | PZP                             | 1.66  |
| Multimerin-1; Platelet glycoprotein Ia*; 155 kDa platelet multimerin          | MMRN1                           | 1.44  |
| Tubulin alpha-4A chain                                                        | TUBA4A                          | 1.39  |
| Alpha-actinin-1                                                               | ACTN1                           | 1.37  |
| Transgelin-2                                                                  | TAGLN2                          | 1.36  |
| Platelet factor 4; Platelet factor 4, short form                              | PF4                             | 1.26  |
| Actin, alpha skeletal muscle; Actin, alpha cardiac muscle 1; Actin, gamma-enteric smooth muscle; Actin, aortic smooth muscle | ACTA1; ACTC1; ACTG2; ACTA2 | 1.25  |
| Peptidyl-prolyl cis-trans isomerase A; Peptidyl-prolyl cis-trans isomerase A, N-terminally processed | PPIA                            | 1.19  |
| Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8 | IGHV4OR15-8                     | 1.11  |
| Profilin-1                                                                    |PFN1                            | 1.10  |
| Tropomyosin alpha-4 chain                                                     | TPM4                            | 1.06  |
| Complement factor H-related protein 1                                         | CFHR1                           | 1.04  |
| Complement C4-A; Complement C4 beta chain; Complement C4-A alpha chain; C4a anaphylatoxin; C4b-A; C4d-A; Complement C4 gamma chain | C4A                             | 1.04  |
| Protein names                                                                 | Gene names                      | LogFC |
|------------------------------------------------------------------------------|--------------------------------|-------|
| Reelin                                                                       | RELN                           | -2.58 |
| Spermatid-associated protein                                                 | SPERT                          | -2.34 |
| Cartilage acidic protein 1                                                   | CRTAC1                         | -2.26 |
| Clathrin heavy chain 1                                                       | CLTC                           | -2.23 |
| Collagen-alpha-3(VI) chain                                                   | COL6A3                         | -2.17 |
| Ig kappa chain V-I region HK101                                               |                                | -1.71 |
| Hemoglobin subunit delta                                                     | HBD                            | -1.62 |
| ADP-ribosylation factor 1;ADP-ribosylation factor 3;ADP-ribosylation factor 5;ADP-ribosylation factor 4 | ARF1;ARF3;ARF5;ARF4            | -1.62 |
| Apolipoprotein F                                                             | APOF                           | -1.57 |
| Solute carrier family 2, facilitated glucose transporter member 1            | SLC2A1                         | -1.53 |
| Transferrin receptor protein 1;Transferrin receptor protein 1, serum form   | TFRC                           | -1.52 |
| Ig kappa chain V-I region AU                                                 |                                | -1.48 |
| Integrin alpha-6;Integrin alpha-6 heavy chain;Integrin alpha-6 light chain;Processed integrin alpha-6 | ITGA6                          | -1.47 |
| Band 3 anion transport protein                                               | SLC4A1                         | -1.46 |
| Methionine adenosyltransferase 2 subunit beta                                | MAT2B                          | -1.45 |
| Tenascin-X                                                                   | TNXB                           | -1.43 |
| Syntenin-1                                                                   | SDCBP                          | -1.40 |
| Ig lambda-7 chain C region                                                   | IGLC7                          | -1.35 |
| Ras suppressor protein 1                                                     | RSU1                           | -1.35 |
| Apolipoprotein B-100;Apolipoprotein B-48                                     | APOB                           | -1.34 |
| Lipopolysaccharide-binding protein                                           | LBP                            | -1.31 |
| Carbonic anhydrase 1                                                         | CA1                            | -1.30 |
| Coagulation factor IX;Coagulation factor IXa light chain;Coagulation factor IXa heavy chain | F9                             | -1.28 |
| Carboxypeptidase B2                                                          | CPB2                           | -1.26 |
| CD9 antigen                                                                  | CD9                            | -1.26 |
| Catalase                                                                     | CAT                            | -1.24 |
| Coagulation factor XI;Coagulation factor XIa heavy chain;Coagulation factor XIa light chain | F11                           | -1.24 |
| Ig lambda chain V-I region NIG-64;Ig lambda chain V-I region BL2             |                                | -1.22 |
| Cholesteryl ester transfer protein                                           | CETP                           | -1.18 |
| Cholinesterase                                                              | BCHE                           | -1.18 |
| Zinc finger protein with KRAB and SCAN domains 2                             | ZKSCAN2                        | -1.11 |
| Beta-Ala-His dipeptidase                                                     | CNDP1                          | -1.09 |
| Transforming growth factor-beta-induced protein ig-h3                        | TGFBI                          | -1.08 |
| Erythrocyte band 7 integral membrane protein                                 | STOM                           | -1.05 |
| Ig kappa chain V-II region FR                                                |                                | -1.02 |
Table 10: Comorbidities (all in %)

| Category                                | LYMPHEDEMA (N=37) | LIPEDEMA (N=15) | LYMPHOVASCULAR (N=11) | CONTROL (N=12) |
|-----------------------------------------|-------------------|-----------------|-----------------------|----------------|
| Cancer history                          | 18 (49)           | 2 (13)*         | 2 (18)                | 4 (33)         |
| Venous disease                          | 5 (14)            | 5 (33)          | 0                     | 1 (8)          |
| Cellulitis history                      | 12 (32)           | 2 (13)          | 3 (27)                | 1 (8)          |
| Hypertension                            | 13 (35)           | 5 (33)          | 0                     | 9 (75)*        |
| Dyslipidemia                            | 10 (27)           | 4 (27)          | 2 (18)                | 7 (58)         |
| Cardiac Arrhythmia                      | 3 (8)             | 0               | 0                     | 4 (33)         |
| Coronary artery disease                 | 0                 | 2 (13)          | 2 (18)                | 2 (17)         |
| Congestive heart failure                | 0                 | 2 (13)          | 0                     | 1 (8)          |
| Hypothyroidism                          | 10 (27)           | 2               | 0                     | 1 (8)          |
| Glucose intolerance/ Diabetes mellitus  | 5 (14)            | 2 (13)          | 0                     | 1 (8)          |
| Skin condition                          | 6 (16)            | 0               | 0                     | 3 (25)         |
| Lung disease                            | 4 (11)            | 3 (20)          | 0                     | 1 (8)          |
| Pulmonary hypertension                  | 0                 | 1 (7)           | 1 (9)                 | 0              |
| Anemia/Hematologic disease              | 5 (11)            | 0               | 2 (18)                | 3 (25)         |
| Hyperparathyroidism                     | 2 (5)             | 0               | 0                     | 0              |
| Migraine                                | 4 (11)            | 0               | 0                     | 1 (8)          |
| Irritable bowel syndrome                | 2 (5)             | 0               | 3 (27)                | 0              |
| Ulcerative colitis                      | 1 (3)             | 1 (7)           | 0                     | 2 (17)         |
| Musculoskeletal disease                 | 11 (30)           | 8 (53)          | 0                     | 11 (92)*       |
| Gynecological diseases                  | 5 (14)            | 4 (27)          | 0                     | 1 (8)          |
| Obstructive sleep apnea                 | 4 (11)            | 0               | 1 (9)                 | 0              |
| Anxiety/Depression                      | 13 (35)           | 7 (47)          | 3 (27)                | 0*             |
| Vitamin D deficiency                    | 3 (8)             | 0               | 0                     | 0              |
| Gastric ulcer                           | 7 (19)            | 1 (7)           | 0                     | 0              |
| Gastrointestinal disorders              | 4 (11)            | 4 (27)          | 1 (9)                 | 0              |
| Allergy                                 | 4 (11)            | 0               | 0                     | 4 (33)         |
| Nephrolithiasis/hydronephrosis          | 2 (5)             | 1 (7)           | 2 (18)                | 1 (8)          |
| Neuropathy                              | 8 (22)            | 0               | 1 (9)                 | 1 (8)          |
| Renal disease                           | 2 (5)             | 0               | 1 (9)                 | 1 (8)          |
| Structural Cardiovascular disease       | 4 (11)            | 0               | 1 (9)                 | 0              |
| Macular degeneration                    | 1 (3)             | 1 (7)           | 0                     | 0              |
| Addison's disease                       | 1 (3)             | 0               | 0                     | 0              |
| Esophageal varices                      | 1 (3)             | 0               | 0                     | 0              |
| Pleural effusion                        | 2 (5)             | 0               | 1 (9)                 | 0              |
| Hypogammaglobulinemia                   | 1 (3)             | 0               | 0                     | 0              |
| Deep vein thrombosis by history         | 0                 | 3 (20)          | 0                     | 0              |
| Glaucoma                                | 0                 | 1 (7)           | 0                     | 0              |
| Sarcoidosis                             | 0                 | 1 (7)           | 0                     | 0              |
| Skin cancer                             | 0                 | 1 (7)           | 1 (9)                 | 0              |
| Cardiomyopathy                          | 0                 | 0               | 0                     | 2 (17)         |
All statistical comparisons were with lymphedema and utilized the Fisher Exact test. Unless, designated, there were no statistically significant differences. * p< 0.05 compared to lymphedema.