European Surgical Research

Eur Surg Res 2017;58:180–192
DOI: 10.1159/000461579
Received: November 30, 2016
Accepted after revision: February 9, 2017
Published online: March 17, 2017

Original Paper

High-Fat Diet in the Absence of Obesity Does Not Aggravate Surgically Induced Lymphoedema in Mice

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Keywords
Lymphoedema · High-fat diet · Lymphatic vessel function

Abstract
Background: Lymphoedema represents the cardinal manifestation of lymphatic dysfunction and is associated with expansion of the adipose tissue in the affected limb. In mice, high-fat diet (HFD)-induced obesity was associated with impaired collecting lymphatic vessel function, and adiposity aggravated surgery-induced lymphoedema in a mouse model. The aim of the current study was to investigate whether adiposity is necessary to impair lymphatic function or whether increased lipid exposure alone might be sufficient in a surgical lymphoedema model. ***Methods:*** To investigate the role of increased lipid exposure in lymphoedema development we used a well-established mouse tail lymphoedema model. Female mice were subjected to a short-term (6 weeks) HFD, without development of obesity, before surgical induction of lymphedema. Lymphoedema was followed over a period of 6 weeks measuring oedema, evaluating tissue histology and lymphatic vascular function. ***Results:*** HFD increased baseline angiogenesis and average lymphatic vessel size in comparison to the chow control group. Upon induction of lymphedema, HFD-treated mice did not exhibit aggravated oedema and no morphological differences were observed in the blood and lymphatic vasculature. Importantly, the levels of fibro-adipose tissue deposition were comparable between the 2 groups and lymphatic vessel function was not impaired as a result of the HFD. Although the net immune cell infiltration was comparable, the HFD group displayed an increased infiltration of

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Eur Surg Res 2017;58:180–192
DOI: 10.1159/000461579

macrophages, which exhibited an M2 polarization phenotype. ***Conclusions:*** These results indicate that increased adiposity rather than dietary influences determines predisposition to or severity of lymphedema.

Introduction

The lymphatic system contributes to the maintenance of tissue fluid homeostasis, immune surveillance, and lipid metabolism by transporting lipids taken up by the small intestine, and also plays a critical role in many pathological conditions [1]. Dysfunction of the lymphatic system leads to accumulation of extravasated fluid and macromolecules, a condition commonly recognized as lymphedema. Whereas filariasis, a parasitic mosquito-borne infection, represents the main cause of lymphoedema in developing countries, in developed countries lymphoedema commonly occurs as a post-cancer treatment complication, mainly after surgery for breast cancer or other gynaecological cancers [2].

The clinical manifestations of lymphoedema include swelling and a feeling of heaviness of the affected limb, immune cell infiltration, and fibrosis [3]. The oedematous limbs do not merely contain fluid but also an increased amount of adipose tissue, and surgical removal of adipose tissue improves the course of the disease [4]. Similar findings in experimental models, where lymphoedema results in localized adipose tissue deposition [5], indicate a clear link between impaired lymphatic transport and localized adipose tissue accumulation.

There is increasing evidence that obesity constitutes a risk factor for lymphoedema development [6, 7]. Lower extremity lymphoedema has been reported in patients with significantly elevated body mass index [8], and an increased body mass index was also associated with lymphoscintigraphic findings of compromised lymphatic vessel function in the affected limbs [9]. A comparison of the lymphatic transport in the subcutaneous adipose tissue of obese and lean individuals indicated that the drainage of macromolecules was less efficient in obese adipose tissue than in lean adipose tissue since there was an increase in the lymphatic drainage rate in the postprandial state versus preprandial state in lean adipose tissue but not in the adipose tissue of obese subjects [10]. These data suggest a causal role of obesity in lymphatic insufficiency.

While the lipid transport function of lymphatic vessels was identified as early as the 17th century by Gaspare Asseli [11], the interplay between the lymphatic system and the adipose tissue has been largely overlooked until recently [12]. In a genetic mouse model with a defective lymphatic system, namely Prox-1 heterozygous mice, lymphatic leakage was observed together with late-onset obesity [13]. Importantly, the obesity phenotype could be reversed after Prox-1 expression was specifically rescued in the lymphatic vessels [14], indicating that the lymphatic dysfunction in those mice was responsible for the obesity. In experimental mouse models of diet-induced obesity, chronic high-fat diet (HFD) and obesity resulted in impaired collecting lymphatic vessel function [15] as well as impaired dendritic cell migration to the lymph nodes [16], whereas diet-induced visceral fat accumulation has been reported to lead to atrophy of the mesenteric lymph nodes in obese mice [17]. Furthermore, HFD-induced obesity aggravated inflammation and fibrotic tissue deposition in a murine model of lymphoedema [16].

Lymphatic dysfunction was also found in mouse models of dyslipidaemia. For instance, hypercholesterolaemic mice exhibited lymphatic vessel dysfunction with tissue swelling, lymphatic leakage, decreased lymphatic transport, and abnormal lymphatic morphology [18]. Additionally, recent reports indicate a potential link between impaired lymphatic vessel

function, lipoprotein metabolism, increased plasma cholesterol levels, and enhanced atherosclerosis [19].

In most of the aforementioned studies, obesity was induced by HFD feeding, which then ultimately resulted in impaired lymphatic transport. It has remained unclear, however, whether these effects were due to obesity or due to the HFD itself. The purpose of this study was to examine the effect of HFD alone on the lymphatic vasculature and on the course of experimentally induced lymphedema. We used a murine model of surgically induced secondary lymphedema, using female mice, which do not develop obesity under HFD [17]. HFD alone resulted in a baseline increase in the tissue area covered by blood and lymphatic vessels. Upon lymphoedema induction, both groups exhibited comparable swelling and no significant morphological differences in the blood and lymphatic vasculature were detected. No differences in fibrotic and adipose tissue deposition were observed between the 2 groups and the contractility of collecting lymphatic vessels was not impaired as a result of the diet. Despite the comparable net immune cell infiltration, the HFD group exhibited an increased infiltration of M2-type macrophages in the skin. Together, these results indicate that HFD in the absence of obesity is not sufficient to aggravate surgically induced secondary lymphedema.

Materials and Methods

Mice and Diet

Female C57BL/6 mice were kept under conventional SPF conditions. Female mice were selected based on the relevance of lymphoedema as a post-breast-cancer treatment complication mainly affecting women. Starting at 4 weeks of age, half of the mice were provided ad libitum access to standard chow diet (chow; 11% kcal deriving from fat, 31% kcal from protein, and 58% from carbohydrates; Provimi-Kliba, Kaiseraugst, Switzerland) and the other half to HFD (60% kcal deriving from lard [pig fat], 20% kcal from protein, and 20% from carbohydrates; Research Diets Inc., New Brunswick, NJ, USA). Mice were kept on chow/HFD for 6 weeks before being operated, and thereafter until the time of sacrifice. The Cantonal Veterinary Office of Zurich (Kantonales Veterinäramt Zürich) approved the current study (Animal License 225/2013).

Lymphoedema Model

Mice were operated at the age of 10 weeks as previously described [20, 21]. Briefly, mice were anaesthetized with isoflurane (2.5%). A circumferential portion of full-thickness skin, 4–5 mm wide, was excised 2 cm below the base of the tail. Subsequently, the collecting lymphatic vessels running along the lateral tail veins were identified under a dissecting microscope and were excised, sparing the veins. Mice were monitored for 3 consecutive days following the operation and thereafter once weekly until the termination of the study.

In vivo Imaging of Lymphatic Vessel Contractility

Mice were anaesthetized by intraperitoneal injection of 0.2 mg/kg medetomidine and 80 mg/kg ketamine before imaging. A Zeiss StereoLumar V12 stereomicroscope, adapted for near-infrared imaging, was used for lymphatic imaging [22]. A Harvard Apparatus PHD2000 syringe pump and a custom-made intradermal catheter consisting of PE10 tubing and a 30-g needle were used to inject a bolus of 5 μL of 20 μM of the PEGylated near-infrared dye P20D800 [22] at approximately 1 cm proximal to the tail tip, followed by controlled infusion of an additional 1 μL at a rate of 0.2 μL/min. During the controlled infusion, a 15-min video was recorded 1–2 cm distal to the wound site. On the video recording, a region of interest analysis of fluorescent intensity was performed using Zeiss AxioVision software to evaluate the contractile function of the collecting lymphatic vessels. A custom software in Matlab [23] was used to analyse the frequency (in contractions per min) and amplitude (in % of mean fluorescence intensity) of the contractions. The pumping score was calculated as a product of frequency and amplitude.

Evaluation of Tail Oedema and Histological Analyses

Tail oedema was evaluated with single diameter measurements 4–5 mm distal to the excision margin, using a digital calliper at the indicated time points (prior to operation, 2 weeks and 6 weeks postoperatively).

Cryosections (7 μm) were obtained from a 2- to 3-mm portion of tail skin 1 cm distal to the excision margin and were embedded in OCT. Cryosections were fixed for 2 min in acetone (–20°C) and for 5 min in 80% methanol (4°C), washed in PBS, incubated for 2 h at 4°C in "immunomix" (5% normal donkey serum, 0.2% bovine serum albumin in PBS with 0.3% Triton X) and incubated overnight (4°C) in immunomix with the following antibodies: goat anti-LYVE-1 (1:600, AngioBio), rat anti-Meca-32 (1:200, BD Bioscience), goat anti-CD45 (1:100, R&D Systems), rat anti-CD68 (1:200, Abcam) or goat anti-CD206 (1:200, R&D Systems). The samples were then incubated with Alexa488- and Alexa594-conjugated secondary antibodies (1:200) and Hoechst 33342 (1:1,000, all from Invitrogen), diluted in PBS, for 30 min. Sections were coverslipped with Mowiol (Calbiochem).

Determination of Lipid and Collagen Content

Lipid deposition was evaluated using the BODIPY stain on tail cryosections. In brief, 7-μm-thick sections were fixed with 4% PFA for 15 min, washed and incubated with BODIPY 493/503 (1:2,000, Life Technologies) and Hoechst 33342 (Invitrogen) diluted in Dako diluent (Dako) for 30 min, and then washed again and mounted with Mowiol.

Collagen content was evaluated on paraffin sections stained with picrosirius red [21]. Tail samples (3- to 4-mm thick cross-sections) were obtained 0.5–1 cm distal to the excision margin, fixed in 4% PFA for 4 h at 4°C and then decalcified for 7 days using EDTA 0.5 M, exchanged daily. Tissues were embedded in paraffin and 5-μm-thick sections were obtained. After deparaffinization and rehydration, the sections were stained with picrosirius red (Sigma-Aldrich) for 1 h and then washed in acidified water (0.5% acetic acid in water). Sections were cleared with xylol, drained, and then mounted with Eukitt medium (Fluka).

Morphometric and Morphologic Analyses

Immunofluorescence stains of tail skin sections were examined with an Axioskop 2 mot plus microscope (Carl Zeiss) equipped with an Illuminator HXP 120 and an AxioCam MRc camera, and 5–6 images per tissue section were acquired with a Plan Apochromat ×10/0.45 NA objective. Picrosirius images were obtained using an EC Plan-Neofluar ×2.5/0.075 objective (Axioskop 2 mot plus) or a Panoramic Slide Scanner 250 BF Flash (3D Histech, Ramsey, NJ, USA) equipped with a Brightfield CIS 3CCD VCC-F52U25CL with a ×20/0.8 NA objective, acquiring and evaluating 1–3 images per tissue section. Morphometric analysis of the vessels, immune cells, and collagen content was performed with ImageJ software.

Statistical Analyses

All data represent mean ± SD and sample sizes and the statistical analyses are indicated in the Figure legends. Means of 2 groups were compared with a two-tailed Student *t* test; a Welch correction was applied in case of unequal variances. Weight gain data were analysed with a 2-way ANOVA. Analyses were performed using GraphPad Prism V6.0 (GraphPad Software, San Diego, CA, USA), SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) or Matlab version 7.12.0.635 R2011a (Mathworks, Inc.). $p < 0.05$ was accepted as statistically significant.

Results

HFD without Obesity Is Not Sufficient to Aggravate Swelling during Lymphoedema Development

To study the effects of HFD in the absence of obesity, 4-week-old female C57BL/6 mice were fed with regular mouse chow or HFD for a period of 6 weeks before surgical induction of lymphedema, and were then kept on the corresponding diets until the end of the experiments (Fig. 1a). At 10 weeks of age, before lymphoedema was induced, the mice under HFD exhibited a modest weight increase compared to chow-fed mice (chow: 19.9 ± 0.9 g, $n = 5$; HFD: 21.7 ± 1.2 g, $n = 5$; $p = 0.0299$) as well as elevated serum triglyceride levels (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000461579). At 12 weeks of age (2 weeks postoperatively), the weight was not significantly different between the 2 groups (chow: 20.1 ± 0.6 g, $n = 7$; HFD: 21.2 ± 1.7 g, $n = 6$; $p = 0.1680$), whereas HFD mice were significantly heavier at 6 weeks after the operation (chow week 6: 21.3 ± 1.1

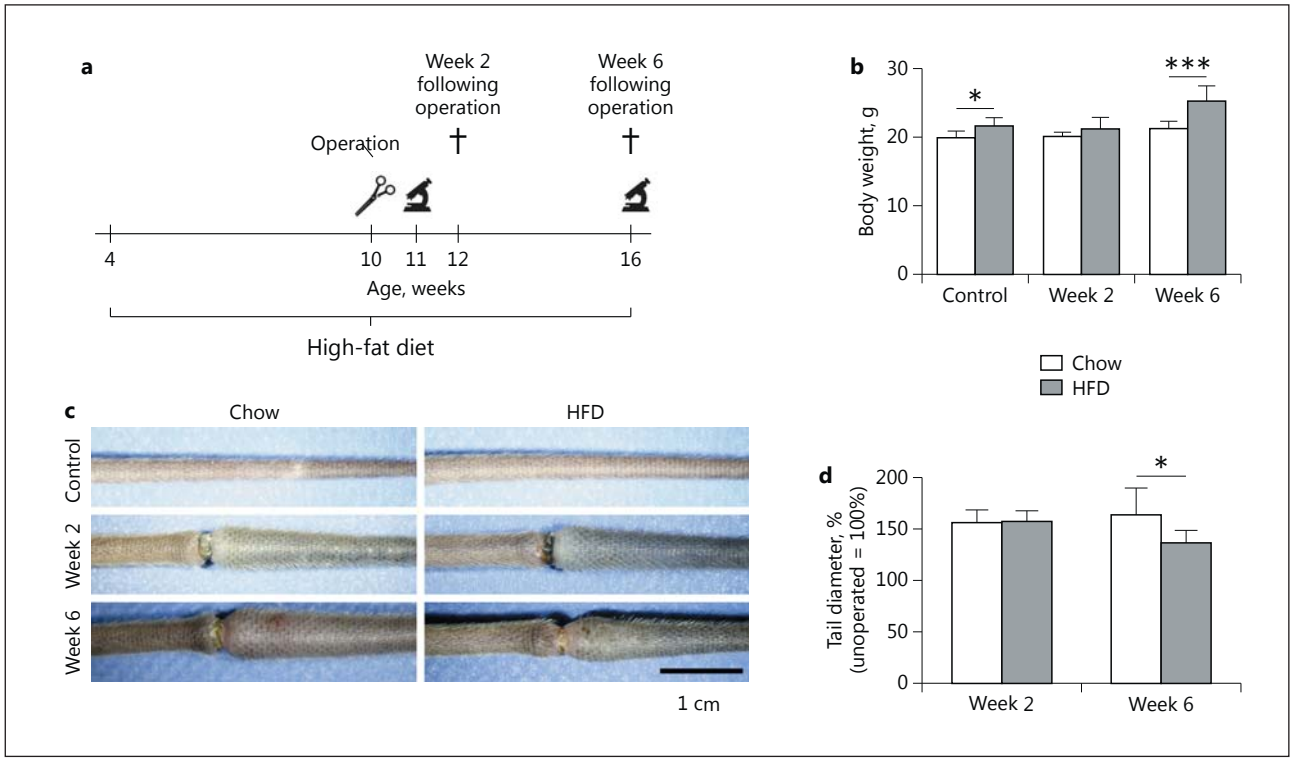

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Fig. 1. Lymphoedema development in an experimental murine tail model. **a** Schematic representation of study design: mice at the age of 4 weeks were placed on HFD or chow diet. At 10 weeks of age, mice were operated and then examined 1, 2 or 6 weeks postoperatively. **b** Body weight measurements of chow- and HFD-treated groups over the study period. **c** Representative photographs of mouse tails of the chow and HFD groups before and after lymphoedema induction. **d** Percent change in tail diameters of chow and HFD groups during the course of lymphoedema development. The means of the 2 groups (chow-HFD) were compared with a two-tailed Student *t* test, applying Welch correction when necessary. Asterisks indicate statistical significance (* $p < 0.05$, *** $p < 0.001$). $n = 5$–8 per group. HFD, high-fat diet.

g, $n = 8$; HFD: 25.2 ± 2.4 g, $n = 8$; $p = 0.0008$; Fig. 1b). Comparison of oedema development was based on the evaluation of tail diameters, measured 2.5 cm distal to the tail base (Fig. 1c). At 2 weeks postoperatively, the increase in tail diameters in comparison to the preoperative values was comparable between the 2 groups (chow: 156.2 ± 12.8%, $n = 7$; HFD: 157.3 ± 10.4%, $n = 5$; $p < 0.0001$; Fig. 1d). However, at 6 weeks postoperatively, the tail thickness increase was significantly lower in the HFD group (chow: 163.9 ± 26.5%, $n = 8$; HFD: 136.1 ± 12.4%, $n = 6$; $p = 0.0353$; Fig. 1d), probably because of the slightly increased baseline tail thickness observed in the HFD mice (chow: 3.1 ± 0.1 mm, $n = 5$; HFD: 3.4 ± 0.2 mm, $n = 5$; $p = 0.0026$; data not shown) that was likely due to increased adipose tissue accumulation.

HFD without Obesity Does Not Increase Fibro-Adipose Tissue Accumulation in Lymphedema

Fibrosis and adipose tissue accumulation are hallmarks of lymphoedema development [3]; therefore, we next investigated whether there were any differences of collagen or lipid deposition between the chow and HFD groups. Fibrosis was evaluated on tail tissue cross-sections by picrosirius red staining that depicts collagenous tissue (Fig. 2a). Quantification of stained sections revealed an increased collagen deposition in the tails of the unoperated HFD group as compared to the unoperated chow group (chow: 0.2774 ± 0.09 mm^2, $n = 5$; HFD:

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DOI: 10.1159/000461579

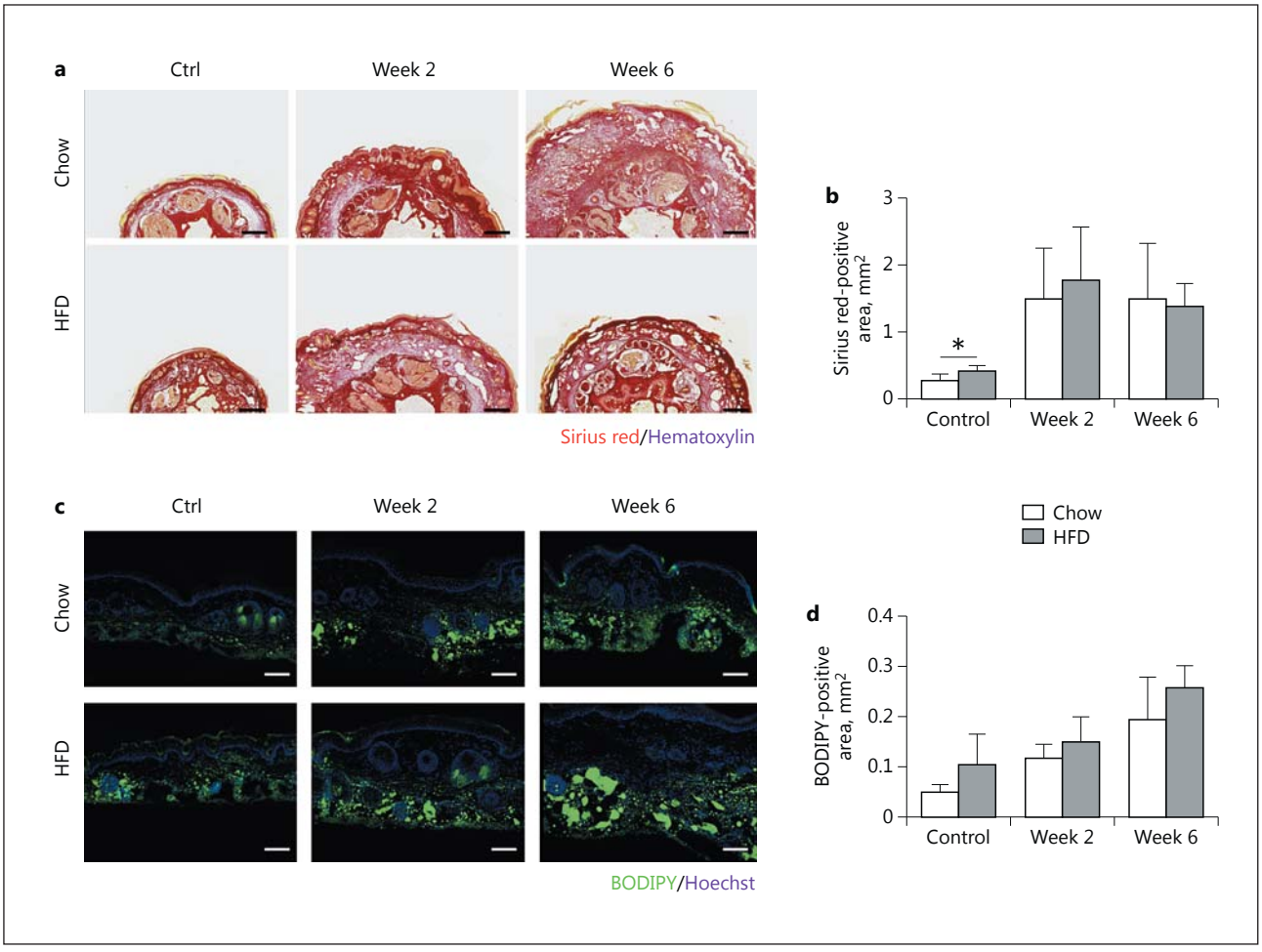

Fig. 2. Fibro-adipose tissue accumulation during lymphoedema development. **a** Picrosirius red and haematoxylin stains of 5-µm-thick tail paraffin sections indicate the collagen deposition. **b** Quantification of collagen deposition (picrosirius red stains; in mm^2) in the area between the epidermal-dermal basement membrane and the muscle fascia. **c** BODIPY stains of 7-µm-thick cryosections showed lipid deposition during lymphoedema development. **d** Quantification of BODIPY stains, expressed as percent of total tissue area stained. The means of the 2 groups were compared with a two-tailed Student *t* test, applying Welch correction when necessary. Asterisk indicates statistical significance (* $p < 0.05$). $n = 5–8$ per group. Scale bars: 500 µm (**a**), 100 µm (**c**). For colours, see online version.

0.4216 ± 0.08 mm^2, $n = 5$; $p = 0.0261$; Fig. 2b), but no difference was seen between the groups at 2 and 6 weeks after lymphoedema surgery. Quantification of lipid deposition using the BODIPY lipid stain showed a minor, nonsignificant increase in lipid accumulation in the tail skin of HFD mice at all investigated time points (Fig. 2c, d).

HFD without Obesity Does Not Affect Blood or Lymphatic Vessel Morphology in Lymphedema

Lymphatic vascular remodelling occurs during lymphoedema development and is associated with the severity of the disease [21]. We next investigated blood and lymphatic vessels in tissue sections stained for the lymphatic marker LYVE-1 and the blood vascular marker Meca-32 before and after lymphoedema induction (Fig. 3a). In unoperated mice, 6 weeks of HFD led to an increase in the tissue area occupied by blood vessels (chow: 1.6 ± 0.4%, $n = 5$;

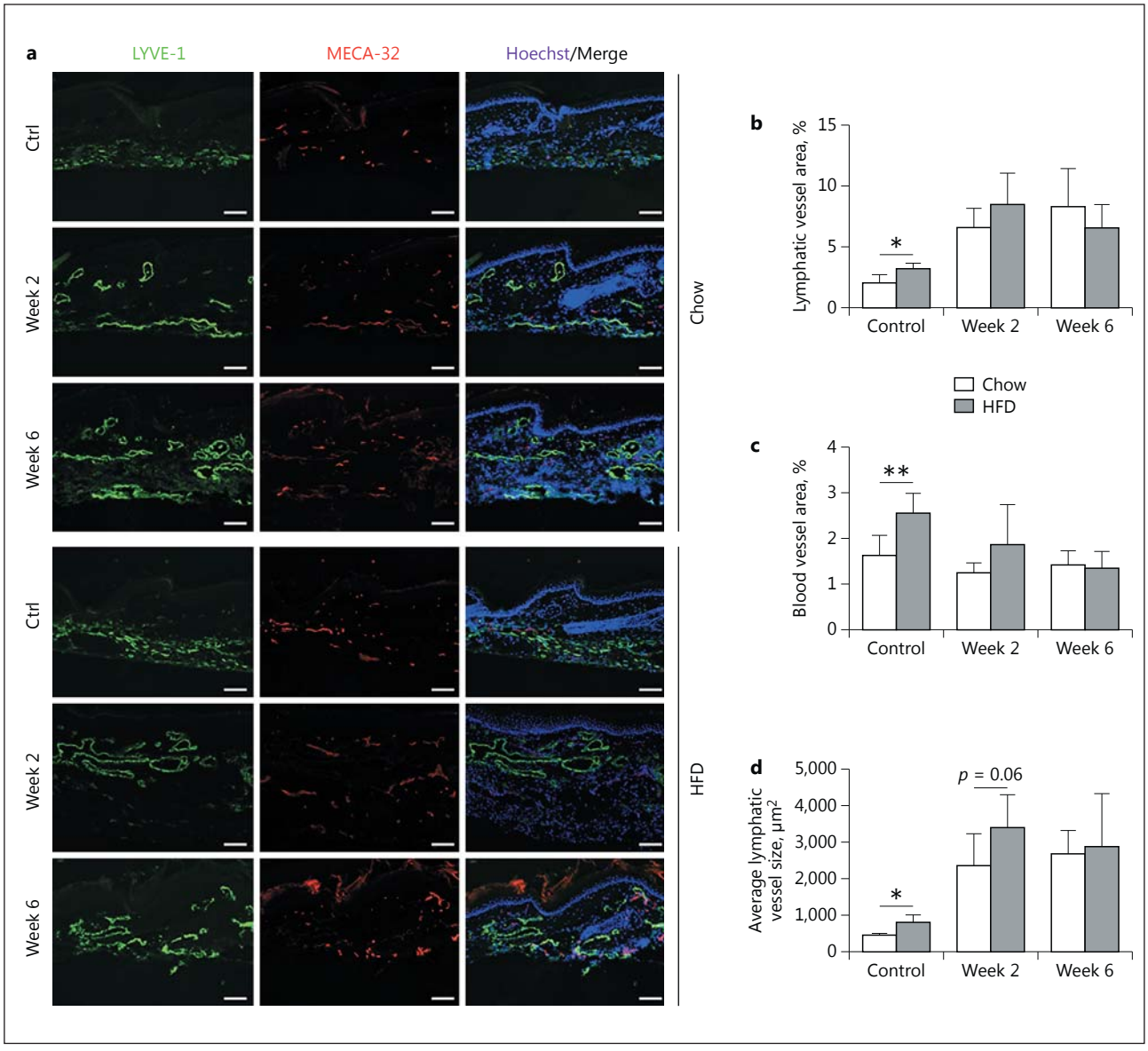

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Fig. 3. Lymphatic vessel remodelling in lymphedema. **a** Seven-micrometre-thick cryosections of tail samples from chow and HFD mice were stained for lymphatic (LYVE-1; green) and blood (Meca-32; red) vessels. Hoechst nuclear stain in blue. **b**, **c** Quantification of LYVE-1-positive and Meca-32-positive tissue area in percent of total area. **d** The average lymphatic vessel size was calculated by dividing the LYVE-1-positive area (including lymphatic vessel lumen) by the total number of lymphatic vessels. The means of the 2 groups (chow and HFD) were compared with a 2-tailed Student *t* test, applying Welch correction when necessary. Asterisks indicate statistical significance (* $p < 0.05$, ** $p < 0.01$). $n = 5–6$ per group. Scale bars = 100 µm. For colours, see online version.

HFD: 2.6 ± 0.4%, $n = 5$; $p = 0.0095$) and by lymphatic vessels (chow: 2.1 ± 0.7%, $n = 5$; HFD: 3.2 ± 0.5%, $n = 5$; $p = 0.0127$; Fig. 3b, c) and the average size of lymphatic vessels was significantly larger (chow: 451.6 ± 46.4 µm^2, $n = 5$; HFD: 802.0 ± 199.2 µm^2, $n = 5$; $p = 0.0154$; Fig. 3d). Upon induction of lymphedema, the blood vascular coverage did not significantly differ between the 2 groups, but the lymphatic vessel area was increased in both groups, as compared to unoperated mice. There were no significant differences between the HFD and

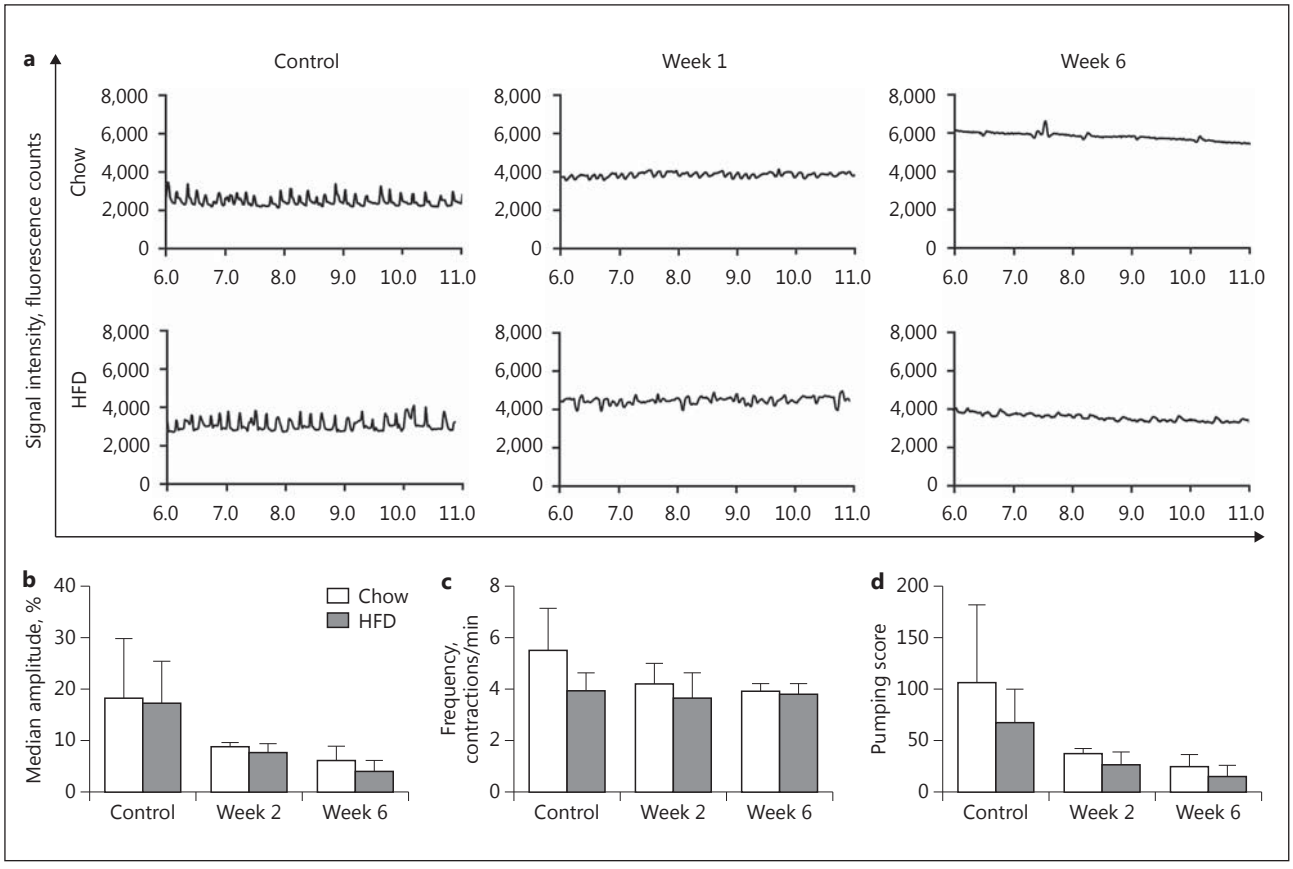
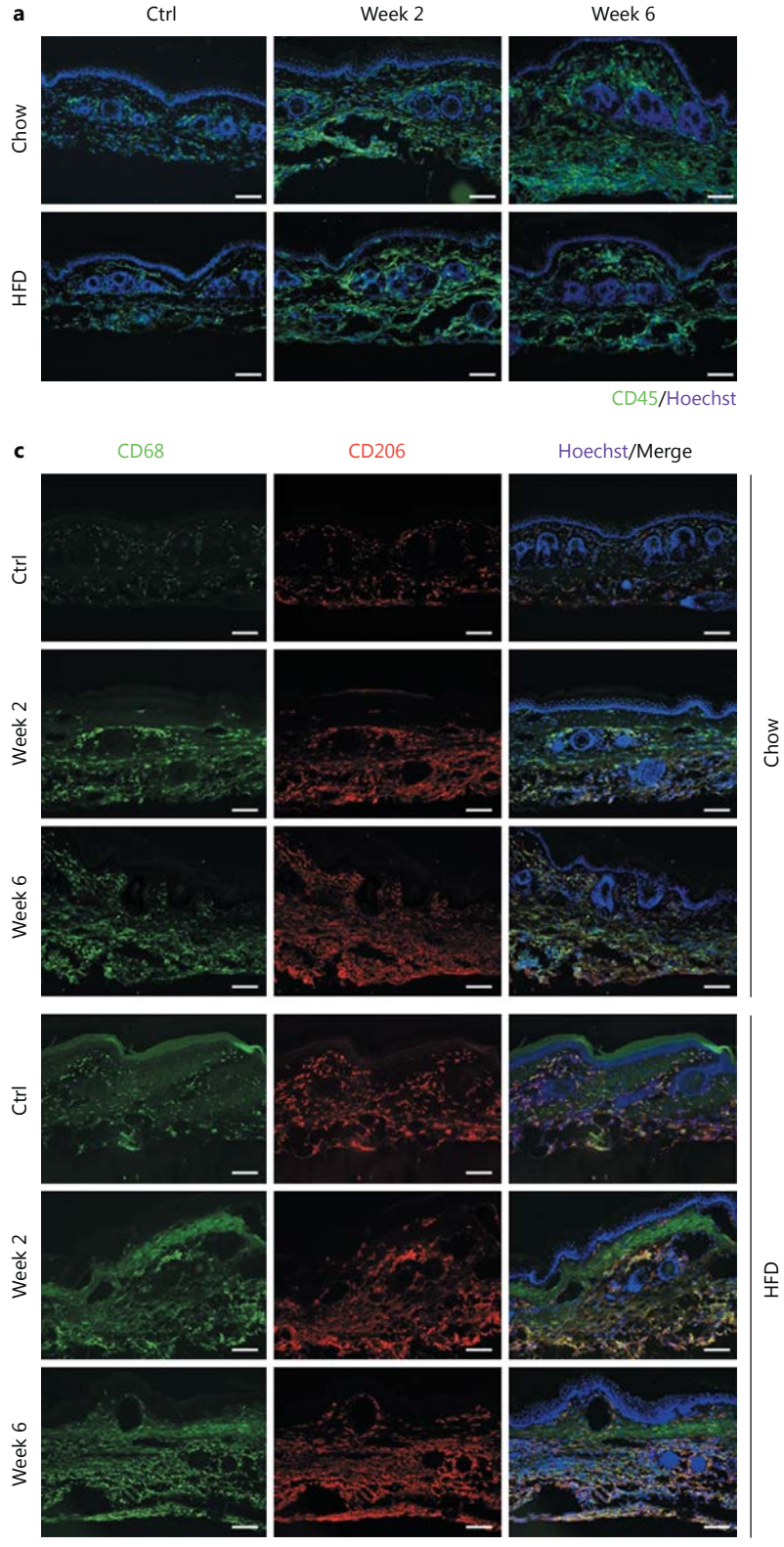


Fig. 4. Quantitative evaluation of collecting lymphatic vessel contractility of chow- and HFD-fed mice. **a** Representative contractility patterns detected in unoperated tails and in tails 1 week and 6 weeks after lymphoedema surgery, indicating a lower amplitude during lymphoedema development without major differences between the chow and HFD groups. Quantification of the contraction amplitude (**b**), contraction frequency (**c**), and pumping score (**d**; amplitude × frequency) revealed no major changes between the 2 groups. Means of 2 groups (chow-HFD) were compared with a two-tailed Student *t* test. *n* = 4–7 per group.

chow group (Fig. 3a–c). There was a trend towards larger lymphatic vessels in the HFD group 2 weeks postoperatively (chow: 2,373.0 ± 855.2 μm^2, *n* = 6; HFD: 3,411 ± 889.1 μm^2, *n* = 6; *p* = 0.0664; Fig. 3d), whereas there were no major differences after 6 weeks.

Collecting Lymphatic Vessel Contractility in Lymphoedema Is Not Affected by HFD

We next investigated the functionality of the collecting lymphatic vessels before and after induction of lymphoedema with video acquisitions of the lymphatic vessel transport of an infrared tracer that was injected near the tip of the tail. We employed a region of interest analysis on the collecting lymphatic vessels in an area 1–2 cm distal to the operation site to quantify the contraction amplitude and frequency [23]. The contraction patterns in unoperated mice as well as 1 week and 6 weeks following operation were similar in both groups (Fig. 4a). While the contraction amplitude at 1 week and 6 weeks postoperatively was lower than in unoperated mice, quantitative analyses did not reveal any significant changes in the amplitude or the contraction frequency between the 2 diet groups at any of the investigated time points (Fig. 4b, c). Consequently, there were no major differences in the pumping score, calculated as the product of contraction amplitude and frequency, between the 2 groups (Fig. 4d).



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HFD Promotes M1 and M2 Macrophage Infiltration into Skin

Skin inflammation is one of the main characteristics of lymphedema. We first investigated the immune cell infiltration using immunofluorescence stains for CD45. The extent of CD45-positive cell infiltration increased during lymphoedema development but was comparable in both groups at all time points (Fig. 5a, b). Evaluation of macrophage infiltration using immunostains for CD68 revealed a significantly increased presence of CD68+ cells (expressed as % area positive for CD68) in the HFD-fed group without operation (chow: 1.50 ± 1.0%, n = 5; HFD: 6.2 ± 2.6%, n = 5; p = 0.0052). At 2 and 6 weeks, macrophage infiltration was further increased in both groups but was always significantly higher in the HFD group (chow week 2: 5.0 ± 2.4%, n = 6; HFD week 2: 11.2 ± 2.3%, n = 5; p = 0.0021; chow week 6: 5.7 ± 2.6%, n = 5; HFD week 6: 11.7 ± 3.2%, n = 5; p = 0.0117; Fig. 5c, d). Further characterization of the macrophage populations, staining for M2-polarized macrophages using the marker CD206, indicated a strong, persistent infiltration of CD206+ macrophages in unoperated HFD mice (chow: 1.8 ± 0.4%, n = 5; HFD: 5.1 ± 0.9%, n = 5; p < 0.0001), which was maintained 2 and 6 weeks postoperatively (chow week 2: 1.8 ± 0.8%, n = 6; HFD week 2: 5.7 ± 1.4%, n = 5; p = 0.0002; chow week 6: 2.6 ± 1.3%, n = 5; HFD week 6: 8.4 ± 4.1%, n = 5; p = 0.0302; Fig. 5c, e). Evaluation of the M1/M2 ratio on a transcriptional level using CD11c and iNOS as M1 markers and CD206 as M2 markers revealed no differences in the macrophage infiltrate preoperatively or during the early stage of lymphoedema development but a striking increase in the M1/M2 ratio 6 weeks postoperatively (CD11c/CD206 chow: 3.0 ± 1.3, HFD: 10.3 ± 2.2, p = 0.0013; iNOS/CD206 chow: 0.6 ± 0.1, HFD: 6.9 ± 0.9, p = 0.0006; see online suppl. Fig. 2).

Discussion

Lymphoedema is the ultimate consequence of lymphatic dysfunction and is usually a iatrogenic complication following surgical cancer treatment. Recently, it was proposed that obesity might aggravate lymphoedema [16]. Indeed, several studies in patients and experimental rodent models of obesity and metabolic syndrome revealed that obesity is related to impaired lymphatic transport capacity, aggravated inflammation, and increased fibrosis [8–10, 15, 16]. While the aforementioned human studies suggested an association between obesity and impaired lymphatic function, experimental rodent models revealed that diet-induced obesity caused lymphatic insufficiency [15]. However, these experimental studies did not identify whether the HFD or the increased adiposity caused the impairment of lymphatic function. In the present study, we show that this effect is most likely due to obesity and not to HFD.

In our experimental model, HFD in the absence of obesity induced a moderate but significant increase in the area covered by blood and lymphatic vessels in the skin and in the

Fig. 5. Evaluation of the immune cell infiltrate in lymphoedema indicates increased M2 macrophage infiltration in the HFD group. **a** Immunofluorescence stains of 7-µm-thick cryosections for the pan-hematopoietic marker CD45 (green). Hoechst nuclear counterstain (blue). **b** Quantification of the CD45+ cell infiltrate revealed no major differences between the 2 groups at all examined time points. **c** Immunofluorescence stains for CD68 (green; all monocytes and macrophages) and CD206 (red; M2-like macrophages) in cryosections. **d**, **e** Quantification of the CD68 and CD206 immunostains revealed increased CD68+ macrophage and CD206+ cell infiltrates in the HFD-treated group at all time points. The means of the 2 groups were compared with a two-tailed Student t test, applying Welch correction when necessary. Asterisks indicate statistical significance (* p < 0.05, ** p < 0.01, *** p < 0.001). n = 6 per group. Scale bar = 100 µm. For colours, see online version.

average lymphatic vessel size in unoperated mice. Indeed, HFD has been reported to induce angiogenesis in the skeletal muscle and to activate angiogenic pathways in blood capillaries [24]. At week 2 after lymphoedema induction, the lymphatic vessel size was increased in both groups, in line with studies showing that lymphatic remodelling occurs as a response to increased tissue oedema [21, 25] but no differences were observed between the 2 groups. Importantly, HFD did not result in any changes in the deposition of fibrotic and adipose tissue, 2 hallmarks of lymphedema, and the contractility of collecting lymphatic vessels was not further impaired as a result of the diet. Recent work has demonstrated that lymphatic vessel function is protected in obesity-resistant mice in comparison to obesity-prone mice and the functional impairment is associated with significant changes in the perilymphatic inflammation [26].

A major finding of the present study was the alteration of the immune compartment in the lymphoedematous skin under HFD. Despite similar trends for CD45+ hematopoietic compartment cells in both groups over the course of lymphoedema development, mice on HFD had an increased infiltration by CD68+ monocytes/macrophages compared to controls at all examined time points. Although it is well established that adiposity is associated with increased macrophage infiltration [27], it is a novel finding that HFD without obesity suffices to increase macrophage infiltration. Moreover, it is of interest that this increase initially correlated with an increase in CD206+ cells. CD206 is generally considered an alternative activation (M2 macrophage) marker [28]. Alternatively polarized macrophages have known angiogenic properties and this could explain the increased baseline blood vessel coverage observed in the tail skin [29, 30]. Despite a significant increase in M2 macrophage markers, we also found a profound increase in the M1/M2 ratio in the HFD group 6 weeks postoperatively suggesting an even greater increase in the M1 macrophage compartment in the HFD group. This is in line with a recent study where HFD-induced obesity led to an increase in both M1 and M2 macrophages, with an increase in the M1/M2 ratio [31]. We observed a subtle increase in the lipid content of the skin as shown by BODIPY staining; hence, it is plausible that an increase in the skin lipids in HFD-fed mice might contribute to recruiting M2 macrophages or polarizing macrophages towards the M2 phenotype. This increase might be enough to recruit immune cells bearing the scavenger receptor CD36, which is centrally involved in the lipid uptake process [32] and was shown to be critical for M2 polarization of macrophages [33].

Several studies reported that obesity and metabolic syndrome might impair lymphatic function via changing the levels of inflammatory stimuli that might alter endothelial barrier functions or diminish the contractility of lymphatic vessels [34]. For instance, Scallan and colleagues [35, 36] reported that impaired nitric oxide signalling was responsible for lymphatic vascular disruption in type 2 diabetes and that the contractility of collecting lymphatic vessels was improved upon genetic deletion of basal nitric oxide synthase. Metabolic syndrome was also reported to contribute to the impairment of lymphatic contractility in rats fed a high-fructose diet [37]. In our study, in the absence of obesity, we found no major differences in the contractility parameters of the collecting lymphatic vessels upon HFD feeding, indicating that HFD without obesity is not sufficient to impair collecting lymphatic vessel function. These results are in agreement with findings in a randomized controlled trial comparing a low-fat diet (fat intake up to 20% of total energy uptake) with a weight reduction diet (introducing a 1,000 kcal daily deficit) and a normal (uncontrolled) diet in breast cancer-related lymphedema. In this trial, a significant correlation between weight loss and reduction of the arm volume was found, indicating that alternation of the diet composition alone without weight reduction is not sufficient to reduce the volume of the affected limb in breast cancer-related lymphoedema [38, 39].

Together, our results indicate that HFD alone does not render mice more susceptible to an increased extent of surgically induced lymphedema; however, it leads to a distinct immune

cell infiltration in the skin. Our findings indicate that the improvement achieved by dietary intervention trials in lymphoedema patients is likely due to reduced adiposity and not to the content of the diet.

Acknowledgements

We would like to acknowledge Jeannette Scholl for excellent technical assistance and Joachim Hehl from the Scientific Center for Optical and Electron Microscopy (ScopeM) of the ETH Zurich for support with imaging. This work was supported by Swiss National Science Foundation grant 310030B_147087, European Research Council grant LYVICAM, Oncosuisse, Krebsliga Zurich and Leducq Foundation Transatlantic Network of Excellence grant Lymph Vessels in Obesity and Cardiovascular Disease (all to M.D.) and ETH grant (to M.D. and E.G.).

Disclosure Statement

The authors declare no competing financial interests.

Author Contributions

E.G., S.K., and M.D. designed the study, analysed the data and wrote the manuscript; E.G., S.K., S.T.P., K.L., and D.B. performed experiments, collected and analysed data. All authors read and commented on the final version of the manuscript.

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