IMMUNOHISTOCHEMICAL EXAMINATION OF IMMUNE CELLS IN ADIPOSE TISSUE OF RAINBOW TROUT *(ONCORHYNCHUS MYKISS*) FOLLOWING INTRAPERITONEAL VACCINATION

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Abstract

Mammalian perivisceral adipose has been shown to play an important role in the regulation of the peritoneal immune responses. Recently it has been demonstrated that peritoneal antigens are collected by leukocytes within the visceral adipose mass, and a broad range of immunomodulatory genes are differentially expressed in adipose tissue after intraperitoneal vaccination in rainbow trout. To assess the immune cell component in adipose, immunohistochemical analysis was used to examine B-cell, T-cell and antigen presenting cell (APC) numbers and distribution in rainbow trout adipose tissue 24 and 72 h post vaccination in comparison to control fish. The results of this study support previous work on mammals with omental milky spots in naïve fish found to contain APCs and T-cells which then increased in size, number and complexity following vaccination. It suggests that following peritoneal stimulation the visceral adipose mass in fish likely plays an important role in vaccine antigen uptake and presentation by APCs, as well as subsequent T-cell activation and differentiation.

Keywords:

rainbow trout
adipose tissue
immunohistochemistry
cell markers
vaccination
milky spots
APC
T-cell
B-cell

1. Introduction

Oil-adjuvanted vaccines used in aquaculture are injected directly into the peritoneal cavity, which in mammals and fish contains a wide range of immune cells (van Vugt et al., 1996; Rapoport et al., 1999; Williams et al., 2010; Mathis et al., 2013). While the resident cell population can vary between teleost species (Meseguer et al., 1993; Tumbol et al., 2009), the composition in rainbow trout (*Oncorhynchus mykiss*) is dominated by myeloid and lymphocyte cells (Afonso et al., 1997, 1998; Korytář et al., 2013). The injection of vaccines (or other inflammatory agents) into the peritoneal cavity of fish generates a rapid change in composition as well as an increase in the number of cells present (Afonso et al., 1998, 2000; do Vale et al., 2002; Mutoloki et al., 2006; Korytář et al., 2013; Noia et al., 2014; Brietzke et al., 2015), although foreign-body inflammatory reactions can be maintained in the cavity for several months post-vaccination in salmonids (Midtlyng, 1996a, 1996b; Poppe & Breck, 1997; Mutoloki et al., 2004, 2010; Koppang et al., 2005; Evensen et al., 2005; Noia et al., 2014; Villumsen et al., 2015).

Mammalian perivisceral adipose (also referred to as the omentum) has been shown to influence and be influenced by adjacent and embedded lymphocytes, and plays an important role in the regulation of peritoneal immune responses (Rangel-Moreno et al., 2009). The visceral adipose mass is also capable of capturing bacteria and other antigenic particulates from the peritoneal cavity (Cui et al., 2002; Ha et al., 2006; Rangel-Moreno et al., 2009), and promoting immunity against them (Rangel-Moreno et al., 2009). Immune cells and numerous pro-inflammatory, anti-inflammatory and immune-modulating proteins and peptides (including cytokines) have been identified in mammalian adipocytes (Rangel-Moreno et al., 2009; Schäffler & Schölmerich, 2010; Chandra et al., 2011). Omenteal milky spots (MS) contain antigen presenting cells (APCs), T- and B-cells and are thought to play a key role in the transitioning of leukocytes from blood through the omentum to the peritoneal cavity and back (Carlow et al., 2009).

Pignatelli et al. (2014) demonstrated that peritoneal antigens are collected by leukocytes in rainbow trout visceral adipose. These leukocytes transcribe marker genes for different leukocyte subpopulations, and are likely responsible for the secretion of a range of immune cytokines (Pignatelli et al., 2014). The establishment of a mature adipocyte phenotype has been shown to be associated with high activity of immune genes in Atlantic salmon (*Salmo salar*) (Todorčević et al., 2010), and teleost adipocytes have been shown to constitutively express pro-inflammatory cytokines and genes relating to the interferon response (Todorčević et al., 2010; Pignatelli et al., 2014). Alongside evidence demonstrating that rainbow trout visceral adipose is capable of responding to viruses (Pignatelli et al., 2014), bacteria, and pro-inflammatory cytokines (Veenstra et al., 2018), it can be concluded that teleost adipose is an immunologically active tissue. Furthermore, the work of Veenstra et al. (2017) established that a broad range of immunomodulatory genes are differentially expressed in adipose tissue after intraperitoneal (ip) injection of oil-adjuvanted bacterial vaccines and revealed a relationship between adipose tissue immune function and the development of vaccine-induced adhesions.

Since it has been suggested that cellular mechanisms occurring immediately post-vaccination within adipose tissue may contribute to the development of adhesions and potentially be involved in the adaptive immune response (Veenstra et al., 2017), in the present study we assessed immune cell distribution in rainbow trout visceral adipose tissue following injection of an oil-adjuvanted vaccine into the peritoneal cavity, using immunohistochemistry. The results of this work showed that MS in naïve fish contain APCs and T-cells and that following an ip administration of oil-adjuvanted vaccines MS increase in number, size and complexity and are associated with vaccine remnants. Overall the results of this work suggest that the visceral adipose mass in fish likely plays an important role in the uptake and presentation of vaccine antigens and subsequent T-cell activation and differentiation following peritoneal stimulation.

2. Methodology

A total of 12 juvenile rainbow trout weighing approximately 60g (College Mill Trout Farm, Perthshire, U.K.) were maintained in 400L tanks at the University of Aberdeen aquarium facility supplied with recirculating freshwater at 14°C. Fish were fed *ad libitum* daily with commercial pellets (EWOS) and were acclimated for at least two weeks prior to vaccination. All trials were carried out in compliance with the Animals (Scientific Procedures) Act 1986 by a UK Home Office license holder and approved by the ethics committee at the University of Aberdeen. Fish were anaesthetised by immersion with 2-phenoxyethanol (Fluka) and each fish injected intraperitoneally (ip) with either 0.1 mL of phosphate buffered saline (PBS) or a water-in-oil adjuvanted vaccine posterior to the pelvic girdle. The aqueous phase of the vaccine was a formalin-killed whole-cell *A. salmonicida* bacterin (pre-inactivation titre of 1.55 x 10^9 cfu/mL) suspended in BHI Media and provided by Elanco Animal Health Ltd. (Victoria, P.E.~~I.~~, Canada) while the oil phase was comprised of Montanide™ ISA 761 VG (Seppic, France). The water-in-oil emulsions was prepared at a 70:30 oil:water ratio 48h prior to vaccination using a high shear mixer (IKA Ultra Turrax Tube Drive) and was tested for stability prior to use.

Visceral adipose located around the internal organs was harvested from freshly killed trout (n=3 per treatment group per time point) at 24 and 72 h post injection (hpi). These timings were chosen based on the previous study of Veenstra et al. (2017), where the transcript response of immune genes was studied in adipose tissue at 3, 14 and 28 days post-vaccination. In that study gene modulation was already maximal at day 3 in the majority of cases, and so here that timing was included together with an earlier time point to assess whether changes were occurring before this. The tissue was stored in Bouin’s Solution (Sigma) for 18 h, washed 3x in PBS, then left in PBS for 3-5 h. Samples were then stored in 70% ethanol (Sigma) before being embedded in paraffin and sectioned at 5μm onto silane-coated glass slides (Microscopy and Histology Core Facility, University of Aberdeen). Immunohistochemistry for each antibody (Table 1) was performed using reagents from the REAL Dako Envision detection kit (Dako UK Ltd) using a Dako autostainer (Dako) as described previously (Alnabulsi et al., 2017) at the Department of Pathology, NHS Grampian Biorepository (Aberdeen, UK). The antibodies used included a B (IgM) and T (CD3) cell marker, and two markers of antigen presenting cells (APCs), MHC-II and CLEC4T1. In the case of the APC markers CLEC4T1 is related to DC-SIGN (see discussion). Primary antibody dilutions used for immunohistochemistry are described in Table 1. The sections were evaluated by light microscopy using a Zeiss Axioscop 40 (Microscopy and Histology Core Facility, University of Aberdeen).

Table 1: Antibodies used for immunohistochemical analysis.

Antibody	Type	Dilution	Dilutant	Reference/Source
IgM (4C10)	Monoclonal	1:5	Dako antibody dilutant	Thuvander et al., 1987
CD3-γδ	Monoclonal	1:15	Dako antibody dilutant	Vertebrate Antibodies Ltd
MHC-IIβ	Rabbit polyclonal	1:200	PBST*	Vertebrate Antibodies Ltd
CLEC4-T1	Rabbit polyclonal	1:500	Dako antibody dilutant	Johansson et al., 2016

* PBST = Phosphate Buffered Saline with Tween 20 (Sigma)

3. Results

The number of CLEC4T1, MHC-II, CD3 and IgM positive cells was found to vary between treatment groups and time points. These results indicate that changes in expression and distribution of APCs, T- and B-cells occur in rainbow trout adipose tissue following vaccination.

CLEC4T1 staining was observed in areas analogous to the centre of clostridial MS located on the periphery of the omentum tissue and in cells encircling apoptotic adipocytes- crown like structures (CLS) in the naïve fish (Fig. 1A & 1C). At 24 and 72 hpi, large quantities of CLEC4T1 positive cells were observed infiltrating the adipose tissue, primarily associated with areas of vaccine-induced cellular damage (Fig. 1B) as well as strongly presenting within newly developed clostridial MS within the adipose tissue (Fig. 1D).

MHC-II positive cells in naïve fish was observed in macrophage-like cells located within adipocyte junctions and in some cell clusters (Fig. 2A & 2C), but were not associated with CLS (Fig. 2C). The largest amount of anti-MHC-II staining was observed at 24 hpi in the vaccinated group and was associated with granulomatous cell clusters and areas of vaccine-infiltration (Fig. 2B). By 72 hpi the quantity of MHC-

II positive cells decreased in the vaccinated group, but staining of small clusters of mononuclear cells within the adipose tissue and associated with MS were still apparent (Fig. 2D).

CD3 was detectable in the adipose tissue of naïve fish, in the cytoplasm of single mononuclear cells found in cell clusters within adipose (Fig. 3A), and in some structures analogous to clostridial MS found on the periphery of adipose tissue (Fig. 3C). In vaccinated fish at 24 hpi the staining appeared much stronger, and was present in an increased number of peripheral MS, as well as newly developed clostridial MS structures throughout the tissue. (Fig. 3B). By 72 hpi staining was still clearly present within MS of vaccinated fish, although weaker than seen in the 24 hpi fish. In vaccinated fish, CD3 positive- MS were associated with CLS (Fig. 3D). The increase in CD3 positive stained structures in cells located within milky spots can be observed in greater detail in Figure 4.

IgM positive cells were not found in the control fish (Fig. 5A & 5C). However, following vaccination cells staining positive for IgM could be observed within adipocyte junctions at 24 hpi (Fig. 5B). Staining was still present but weaker at 72 hpi in individual cells, occasionally associated with MS (Fig. 5D). Staining was also present within blood vessels in the vaccinated groups, presumed to be soluble IgM in the blood (Fig. 5D).

4. Discussion

As teleost adipose has been found capable of sequestering antigens from the peritoneal cavity (Pignatelli et al., 2014), and immune-related genes were transcriptionally upregulated as early as 72 h after ip vaccination (Veenstra et al., 2017), in this study we aimed to further characterize the relationship between vaccine-induced stimulation of the peritoneal cavity and adipose immune cell response up to 72 h post injection via immunohistochemical analysis. The results of this study were found to be broadly similar to what has been described previously in regards to mammalian adipose clostridial milky spots (MS). MS associated with peritoneal adipose tissue (omentum) have been described in a number of species (Mixter, 1941) including fish (Pignatelli et al., 2014). They have been shown to contain macrophages, APCs, T- and B-cells (Carlow et al., 2009) and to have important biological functions within the peritoneal cavity (Beelen, 1991; Shimotsuma et al., 1993; Takemori et al., 1995, Lenzi et al., 1996; van Vugt et al., 1996) and omentum (Carlow et al., 2009; Rangel-Moreno et al., 2009), acting as a gateway through which circulating cells, antigens, particulates and pathogens are collected from the peritoneal cavity to promote a variety of immune responses (Beelen et al., 1980a, 1980b; Cranshaw & Leak, 1990). Following stimulation in mammals, the increases in the number and size of MS occur alongside an influx of leukocytes within MS (van Vugt et al., 1996), as appeared to be happening in the current study. It is worth noting that viral stimulation did not alter the size or number of MS in rainbow trout adipose (Pignatelli et al., 2014).

Dendritic cells (DCs) and macrophages are regarded as the key APCs of the immune system and play an important role in the transition of innate immunity to adaptive immunity. In mammals MS are considered to be the site of origin of peritoneal macrophage precursors (Lee & Lee, 2014). An influx of macrophages into the peritoneal cavity has been described in salmonids following stimulation (Afonso et al., 1998; Jørgensen et al., 2008). C-type lectin (CLEC) domain family 4-T1 is a rainbow trout transmembrane protein thought to be closely related to the well-characterised CLEC4 family protein CD209 / DC-SIGN (Johansson et al., 2016). It, along with MHC class-II proteins are found on DCs/ macrophages and help present extracellular antigens to CD4 positive cells and to promote the rapid activation of T- and B-cells (Carlow et al., 2009). The lack of MHC-II positive staining cells within MS supports previous observations in trout (Pignatelli et al., 2014), however the presence of CLEC4T1 positive cells within these structures demonstrates that APCs (potentially DC or macrophage precursors) are present within MS in naïve fish, in accordance with previous work on mice (Bertola et al., 2012). Additionally, crown-like structures (CLS), described as clusters of macrophages surrounding dead adipocytes in obese mammalian adipose (Murano et al., 2008; Noia et al., 2014), were observed to be strongly CLEC4T1 positive in naïve and vaccinated rainbow trout. As the results in the present study showed that there was little to no overlap in staining patterns of CLEC4T1 and MHC-II, it indicates that within trout adipose tissue these markers are expressed on distinct cell populations at these time points. The key function of immature DCs is capturing and processing antigens which trigger full maturation, and in time leads to the assembly of antigen-MHC-II complexes which are capable of stimulating T-cells (Banchereau & Steinman, 1998; Geijtenbeek et al., 2000; Engering et al., 2002). As it has been demonstrated that bacteria can stimulate DC maturation (Sallusto & Lanzavecchia 1995; Winzler et al., 1997), it is likely that in teleosts APCs preferentially begin production/maturation of CLEC4T1 to facilitate ingestion and presentation of foreign substances with MHC-II complexes playing a larger role at a later time point than studied here.

Lymphocytes are the second major cellular component of normal mammalian MS (Shimotsuma et al., 1991, 1993; Krist et al., 1995). More recent studies (Rangel-Moreno et al., 2009; Carlow et al., 2009) show that the omentum can support the activation of CD4 and CD8 positive lymphocytes and mount T cell-dependent B-cell responses to peritoneal antigens. CD3 is part of the T-cell receptor complex on the cell surface which aids activation of naïve T cells (Guy & Vignali, 2009) and is in rainbow trout considered a good pan-T-cell marker (Leal et al., 2016). The present study reveals the presence of CD3 positive cells in clostridial MS on the periphery of adipose tissue in naïve fish, in distinct areas separate to CLEC4T1 positive cells within MS. An increase in staining intensity was observed at 24 hpi (which reduced by 72 hpi) in MS, which supports work in mammals showing that the omentum effectively operates as a site for early antigen presentation, with a rapid turnover of lymphocytes (Carlow et al., 2009). As CD3 positive MS were also found to be associated with CLEC4T1 positive CLS, it strongly advocates that following vaccination APCs play a large role in antigen uptake, presentation and subsequent T-cell activation in trout adipose tissue MS.

Immunoglobulin (Ig) M is the most ancient and prevalent Ig in fish. It can be expressed on the surface of B-cells or secreted as an antibody. In this study no evidence of IgM positive staining in milky spots was observed, in agreement with work on rainbow trout by Pignatelli et al. (2014) but in contrast to mammalian studies (Rangel-Moreno et al., 2009). Pignatelli et al. (2014) identified IgM positive cells in the interstitial space between adipocytes within visceral adipose and Ballesteros et al. (2013) found that IgM transcript level could be increased in adipose in response to oral vaccination. The present study found evidence of IgM positive cells in interstitial spaces in naïve fish which increased in number following vaccination.

In conclusion, the immunohistochemical results of this paper show that naïve teleost MS contain APCs (CLEC4T1 positive cells) and T-cells (CD3 positive cells). Following the administration of an ip oil-adjuvanted vaccine, MS in rainbow trout adipose increased in number, size and complexity and may play a significant role in T-cell activation and differentiation via APCs.

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Declarations of interest: none

References

Afonso, A., Ellis, A. E. & Silva, M. T. (1997). The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology, 7*: 335-348.

Afonso, A., Lousada, S., Silva, J., Ellis, A. E. & Silva, M. T. (1998). Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. A light and electron microscopic cytochemical study. *Diseases of Aquatic Organisms, 34*: 27-37.

Afonso, A., Macedo, P. M., Ellis, A. E. & Silva, M. T. (2000). Glycogen granules in resting and inflammatory rainbow trout phagocytes–an ultrastructural study. *Diseases of Aquatic Organisms, 42*: 101-110.

Alnabulsi, A., Swan, R., Cash, B., Alnabulsi, A. & Murray, G. I. (2017). The differential expression of omega-3 and omega-6 fatty acid metabolising enzymes in colorectal cancer and its prognostic significance. *Br J Cancer., 116*(12): 1612-1620. doi: 10.1038/bjc.2017.135.

Ballesteros, N. A., Castro, R., Abos, B., Saint-Jean, S. S. R., Pérez-Prieto, S. I. & Tafalla, C. (2013). The pyloric caeca area is a major site for IgM+ and IgT+ B-cell recruitment in response to oral vaccination in rainbow trout. *PLOS ONE, 8*(6): e66118. https://doi.org/10.1371/journal.pone.0066118

Banchereau, J. & Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature, 392*: 245–252. doi:10.1038/32588

Beelen, R. H. J., Fluitsma, D. M. & Hoefsmit, E. C. M. (1980a). The cellular composition of omentum milky spots and the ultrastructure of milky spot macrophages and reticulum cells. *J. Reticuloendothel. Soc., 28*: 585-599.

Beelen, R. H. J., Fluitsma, D. M. & Hoefsmit, E. C. M. (1980b). Peroxidatic activity of mononuclear phagocytes developing in omentum milky spots. *J. Reticulendothel. Soc., 28*: 601-609.

Beelen, R. H. J. (1991). The greater omentum: physiology and immunological concepts. *Neth J Surg, 43*: 145-149.

Bertola, A., Ciucci, T., Rousseau, D., Bourlier, V., Duffaut, C., Bonnafous, S., Blin-Wakkach, C., Anty, R., Iannelli, A., Gugenheim, J., Tran, A., Bouloumié, A., Gual, P. & Wakkach, A. (2012). Identification of adipose tissue dendritic cells correlated with obesity-associated insulin-resistance and inducing Th17 responses in mice and patients. *Diabetes, 61*(9): 2238-47. doi: 10.2337/db11-1274.

Brietzke, A., -Korytář, T., -Jaros, -J., Köllner, B., -Goldammer, T., -Seyfert, H. M. & Rebl, A. (2015). *Aeromonas salmonicida* infection only moderately regulates expression of factors contributing to toll-like receptor signaling but massively activates the cellular and humoral branches of innate immunity in rainbow trout (*Oncorhynchus mykiss*). *Journal of Immunology Research, 2015*(901015): 1-16. doi:10.1155/2015/901015

Carlow, D. A., Gold, M. R. & Ziltener, H. J. (2009). Lymphocytes in the peritoneum home to the omentum and are activated by resident dendritic cells. *J Immunol., 183*(2): 1155-65. doi:10.4049/jimmunol.0900409

Castro, R., Abos, B., Gonzalez, L., Granja, A. G. & Tafalla, C. (2017). Expansion and differentiation of IgM+ B-cells in the rainbow trout peritoneal cavity in response to different antigens. *Developmental and Comparative Immunology, 70*: 119-127.

Chandra, A., Srivastava, R. K., Kashyap, M. P., Kumar, R., Srivastava, R. N. & Pant, A. B. (2011). The anti-inflammatory and antibacterial basis of human omental defense: Selective expression of cytokines and antimicrobial peptides. *PLOS ONE, 6*(5): e20446. https://doi.org/10.1371/journal.pone.0020446

Cranshaw, M. L. & Leak, L. V. (1990). Milky spots of the omentum: a source of peritoneal cells in the normal and stimulated animal. *Arch Histol Cytol, 53*(Suppl): 165-177.

Cui, L., Johkura, K., Liang, Y., Teng, R., Ogiwara. N., … Sasaki, K. (2002). Biodefense function of omental milky spots through cell adhesion molecules and leukocyte proliferation. *Cell Tissue Res., 310*: 321–330.

do Vale, A., Afonso, A. & Silva, M. T. (2002). The professional phagocytes of sea bass (*Dicentrarchus labrax* L.): cytochemical characterisation of neutrophils and macrophages in the normal and inflamed peritoneal cavity. *Fish & Shellfish Immunology, 13*: 183-198.

Engering, A., Geijtenbeek, T. B. H., van Vliet, S. J., … van Kooyk, Y. (2002). The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J. Immunol., 168*(5): 2118 -2126. doi.org/10.4049/jimmunol.168.5.2118

Evensen, Ø., Brudeseth, B.E. & Mutoloki, S. (2005). The vaccine formulation and its role in inflammatory processes in fish--effects and adverse effects. *Developments in Biologicals, 121*: 117–25.

Geijtenbeek, T. B., Torensma, R., van Vliet, S. J. …. Figdor, C. G. (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell, 100*: 575-585.

Guy, C. S. & Vignali, D. A. (2009). Organization of proximal signal initiation at the TCR:CD3 complex. *Immunol. Rev., 232*(1): 7-21. doi: 10.1111/j.1600-065X.2009.00843.x.

Ha, S., Tsuji, M., Suzuki, K., Meek, B., Yasuda, N., Kaisho, T. & Fagarasan, S. (2006). Regulation of B1 cell migration by signals through Toll-like receptors. *Journal of Experimental Medicine, 203*(11): 2541–2550. https://doi.org/10.1084/jem.20061041

Johansson, P., Wang, T., Collet, B., Corripio-Miyar, Y., Monte, M. M., Secombes, C. J. & Zou, J. (2016). Identification and expression modulation of a C-type lectin domain family 4 homologue that is highly expressed in monocytes/macrophages in rainbow trout (*Oncorhynchus mykiss*). *Dev Comp Immunol., 54*(1): 55-65. doi: 10.1016/j.dci.2015.08.005.

Jørgensen, S. M., Afanasyev, S. & Krasnov, A. (2008). Gene expression analyses in Atlantic salmon challenged with infectious salmon anemia virus reveal differences between individuals with early, intermediate and late mortality. *BMC Genomics, 9*: 179. doi:10.1186/1471-2164-9-179

Krist, L. F., Eestermans, I. L., Steenbergen, J. J., Hoefsmit, E. C., Cuesta, M. A., Meyer, S. & Beelen, R. H. (1995). Cellular composition of milky spots in the human greater omentum: an immunochemical and ultrastructural study. *Anat Rec, 241*: 163-174.

Koppang, E. O., Haugarvoll, E., Hordvik, I., Aune, L. & Poppe, T. T. (2005). Vaccine-associated granulomatous inflammation and melanin accumulation in Atlantic salmon, *Salmo salar* L., white muscle. *Journal of Fish Diseases, 28*(1): 13–22. https://doi.org/10.1111/j.1365-2761.2004.00583.x

Korytář, T., Jaros, J., Verleih, M., Rebl, A., Kotterba, G., Kühn, C., Goldammer, T. & Köllner, B. (2013). Novel insights into the peritoneal inflammation of rainbow trout (*Oncorhynchus mykiss*). *Fish & Shellfish Immunology, 35*(4): 1192-1199. https://doi.org/10.1016/j.fsi.2013.07.032

Leal, E., Granja, A. G., Zarza, C. & Tafalla, C. (2016). Distribution of T cells in rainbow trout (*Oncorhynchus mykiss*) skin and responsiveness to viral infection. *PLoS ONE 11*(1): e0147477. https://doi.org/10.1371/journal.pone.0147477

Lee, B-C. & Lee, J. (2014). Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *J Biochimica et Biophysica Acta, Molecular Basis of Disease, 1842*(3): 446-462. https://doi.org/10.1016/j.bbadis.2013.05.017

Lenzi, H. L., Oliveira, D. N., Pelajo-Machado, M., Borojevic, R. & Lenzi, J. A. (1996). Coelom-associated lymphomyeloid tissue (milky spots): site of lymphoid and myelomonocytic cell generation. *Braz J Med Biol Res, 29*: 19-24.

Mathis, D. (2013). Immunological goings-on in visceral adipose tissue. *Cell Metabolism, 17*(6): 851–859. doi:10.1016/j.cmet.2013.05.008

Meseguer, J., Esteban, M. A., Munoz, J., Lopezruiz, A. (1993). Ultrastructure of the peritoneal-exudate cells of seawater teleosts, seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). *Cell and Tissue Research, 273*: 301-307.

Midtlyng, P.J., Reitan, L.J. & Speilberg, L. (1996a). Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (*Salmo salar* L.) against furunculosis. *Fish & Shellfish Immunology, 6*(5): 335–350. doi:10.1006/fsim.1996.0034

Midtlyng, P.J., Reitan, L.J., Lillehaug, A. & Ramstad, A. (1996b). Protection, immune responses and side effects in Atlantic salmon (*Salmo salar* L.) vaccinated against furunculosis by different procedures. *Fish & Shellfish Immunology, 6*(8): 599–613. doi:10.1006/fsim.1996.0055

Mixter, R. L. (1941). On macrophagal foci ("milky spots") in the pleura of different mammals, including man. *Am J Anat, 69*: 159-186.

Murano, I., Barbatelli, G., Parisani, V., Latini, C., Muzzonigro, G., Castellucci, M. & Cinti, S. (2008). Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. (2008). *The Journal of Lipid Research, 49*: 1562-1568. doi: 10.1194/jlr.M800019-JLR200

Mutoloki, S., Alexandersen, S. & Evensen, Ø. (2004). Sequential study of antigen persistence and concomitant inflammatory reactions relative to side-effects and growth of Atlantic salmon (*Salmo salar* L.) following intraperitoneal injection with oil-adjuvanted vaccines. *Fish & Shellfish Immunology, 16*(5): 633–644. https://doi.org/10.1016/j.fsi.2003.10.002

Mutoloki, S., Reite, O.B., Brudeseth, B., Tverdal, A. & Evensen, Ø. (2006). A comparative immunopathological study of injection site reactions in salmonids following intraperitoneal injection with oil-adjuvanted vaccines. *Vaccine, 24*(5): 578–588. doi:10.1016/j.vaccine.2005.08.070

Mutoloki, S., Cooper, G. A., Marjara, I. S., Koop, B. F. & Evensen, Ø. (2010). High gene expression of inflammatory markers and IL-17A correlates with severity of injection site reactions of Atlantic salmon vaccinated with oil-adjuvanted vaccines. *BMC Genomics, 11*(1): 336. https://doi.org/10.1186/1471-2164-11-336

Noia, M., Domínguez, B., Leiro, J., Blanco-Méndez, J., Luzardo-Álvarez, A. & Lamas, J. (2014). Inflammatory responses and side effects generated by several adjuvant-containing vaccines in turbot. *Fish & Shellfish Immunology, 38*(1): 244–254. doi:10.1016/j.fsi.2014.03.020

Pignatelli, J., Castro, R., González Granja, A., Abós, B., González, L., Jensen, L.B. & Tafalla, C. (2014). Immunological characterization of the teleost adipose tissue and its modulation in response to viral infection and fat-content in the diet. *PLoS ONE, 9*(10): e110920. doi:10.1371/journal.pone.0110920

Poppe, T. T. & Breck, O. (1997). Pathology of Atlantic salmon *Salmo salar* intraperitoneally immunised with oil adjuvanted vaccine. A case report *Diseases of Aquatic Organisms, 29*: 219-226.

Rangel-Moreno, J., Moyron-Quiroz, J. E., Carragher, D. M., Kusser, K., Hartson, L., et al. (2009). Omental milky spots develop in the absence of lymphoid tissue-inducer cells and support B and T cell responses to peritoneal antigens. *Immunity 30*: 731–743.

Rapoport, J., Hausmann, M. J. & Chaimovitz, C. (1999). The peritoneal immune system and continuous ambulatory peritoneal dialysis. *Nephron, 81*: 373-380.

Sallusto, F. & Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate antigen to the MHC class II compartment. Downregulation by cytokines and bacterial products. *J. Exp. Med., 182*: 389–400.

Schäffler, A. & Schölmerich, J. (2010). Innate immunity and adipose tissue biology. Trends in Immunology, 31(6): 228–235. https://doi.org/10.1016/j.it.2010.03.001

Shimotsuma, M., Takahashi, T., Kawata, M. & Dux, K. (1991). Cellular subsets of the milky spots in the human great omentum. *Cell Tissue Res, 264*: 599-601.

Shimotsuma, M., Shields, J. W., Simpson-Morgan, M. W., Sakuyama, A., Shirasu, M., Hagiwara, A. & Takahashi, T. (1993). Morpho- physiological function and role of omental milky spots as omentum-associated lymphoid tissue (OALT) in the peritoneal cavity. *Lymphology, 26*: 90-101.

Takemori, N., Hirai, K., Onodera, R., Saito, N. & Namiki, M. (1995). Light and electron microscope study of splenoportal milky spots in New Zealand black mice: comparison between splenoportal milky spots and aberrant spleens. *J Anat, 186*: 287-299.

Thuvander, A., Hongslo, T., Jansson, E. & Sundquist, B. (1987). Duration of protective immunity and antibody titres measured by ELISA after vaccination of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis. *J Fish Dis, 10*: 479-486.

Tumbol, R. A., Baiano, J. C. F. & Barnes, A. C. (2009). Differing cell population structure reflects differing activity of percoll-separated pronephros and peritoneal leucocytes from barramundi (*Lates calcarifer*). *Aquaculture, 292*: 180-188.

Todorčević, M., Škugor, S., Krasnov, A. & Ruyter, B. (2010). Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. *BMC Genomics 11*(1): 39.

van Vugt, E., Van Rijthoven, E. A., Kamperdijk, E. W. & Beelen, R. H. (1996). Omental milky spots in the local immune response in the peritoneal cavity of rats. *Anat Rec, 244*: 235-245.

Veenstra, K. A., Wang, T., Alnabulsi, A., Douglas, A., Russell, K. S., Tubbs, L., Ben Arous, J., Secombes, C. J. (2017). Analysis of adipose tissue immune gene expression after vaccination of rainbow trout with adjuvanted bacterins reveals an association with side effects. *Molecular Immunology, 88*: 89-98. https://doi.org/10.1016/j.molimm.2017.05.026

Veenstra, K. A., Wangkahart, E., Wang, T., Tubbs, L., Ben Arous, J. & Secombes, C. J. (2018). Rainbow trout (*Oncorhynchus mykiss*) adipose tissue undergoes major changes in immune gene expression following bacterial infection or stimulation with pro-inflammatory molecules. *Developmental and Comparative Immunology, 81*: 83-94. DOI: 10.1016/j.dci.2017.11.001

Villumsen, K., Koppang, E. O. & Raida, M. K. (2015). Adverse and long-term protective effects following oil-adjuvanted vaccination against *Aeromonas salmonicida* in rainbow trout. *Fish & Shellfish Immunology, 42*(1): 193–203. https://doi.org/10.1016/j.fsi.2014.09.024

Walker, F. C. & Rogers, A. W. (1961). The greater omentum as a site of antibody synthesis. *Br J Exp Pathol., 42*: 222-31.

Williams, J. C., Wagner, N. J., Earp, H. S., Vilen, B. J., Matsushima, G. K. (2010). Increased hematopoietic cells in the mertk-/- mouse peritoneal cavity: a result of augmented migration. *J. Immunol., 184*: 6637-6648.

Winzler, C., Rovere, P., Rescigno, M., Ricciardi-Castagnoli, P. (1997). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med., 185*: 317–328.

Figure Legends

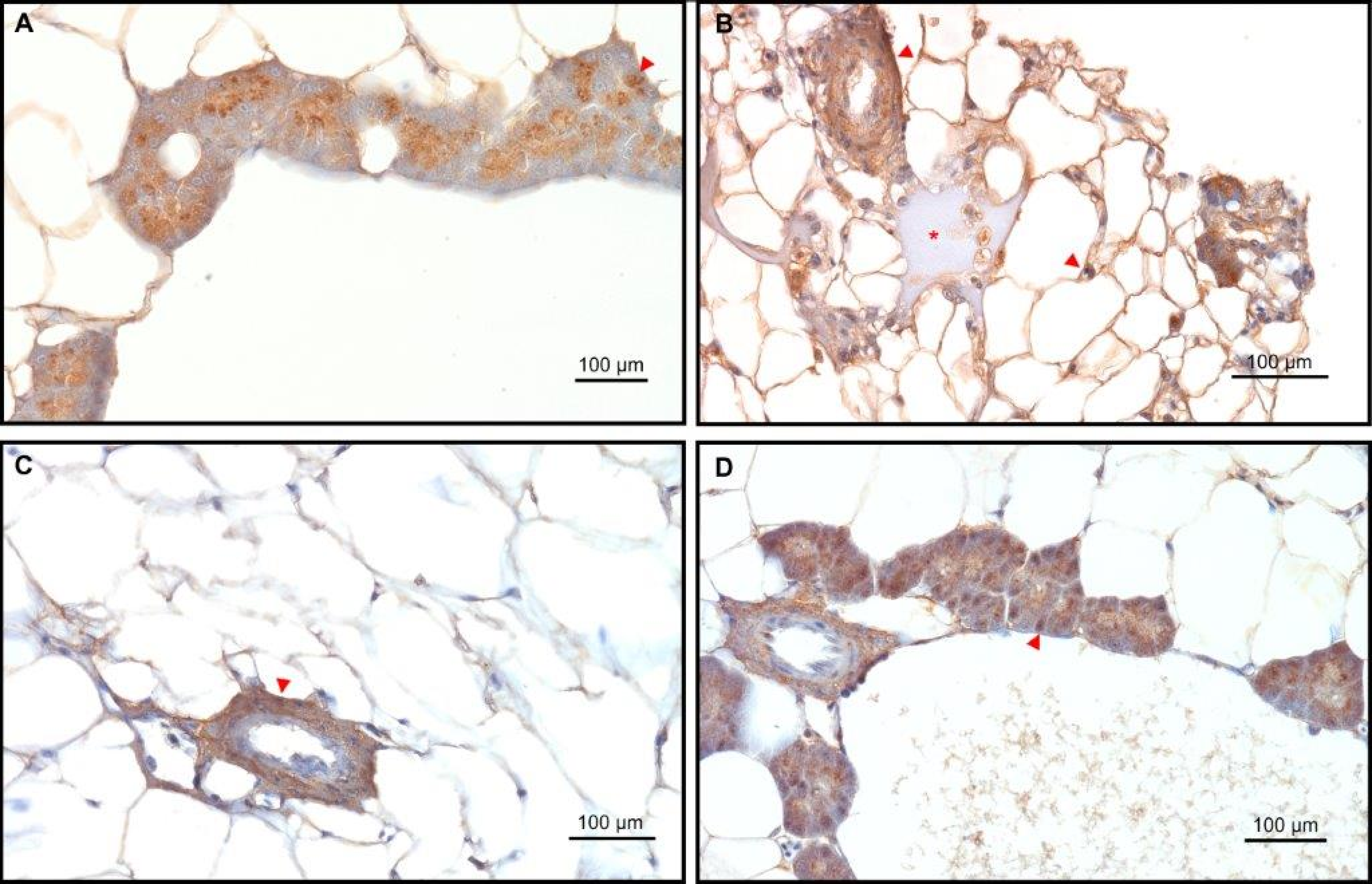
Figure 1: Arrow heads (red) point to representative positive staining of CLEC4T1 in rainbow trout adipose tissue. A: 24 hpi unvaccinated; B: 24 hpi vaccinated; C: 72 hpi unvaccinated; D: 72 hpi vaccinated (star = vaccine remnant).

Figure 2: Arrow heads (red) point to representative positive staining of MHC-II in rainbow trout adipose tissue. A: 24 hpi unvaccinated; B: 24 hpi vaccinated; C: 72 hpi unvaccinated; D: 72 hpi vaccinated (star = vaccine remnant).

Figure 3: Arrow heads (red) point to representative positive staining of CD3-γδ in rainbow trout adipose tissue. A: 24 hpi unvaccinated; B: 24 hpi vaccinated; C: 72 hpi unvaccinated; D: 72 hpi vaccinated.

Figure 4: CD3-γδ positive stained cells in a rainbow trout adipose tissue milky spot at 24 h post-vaccination.

Figure 5: Arrow heads (red) point to representative positive staining of IgM in rainbow trout adipose tissue. Arrow head (black) show staining in blood vessels. A: 24 hpi unvaccinated; B: 24 hpi vaccinated; C: 72 hpi unvaccinated; D: 72 hpi vaccinated.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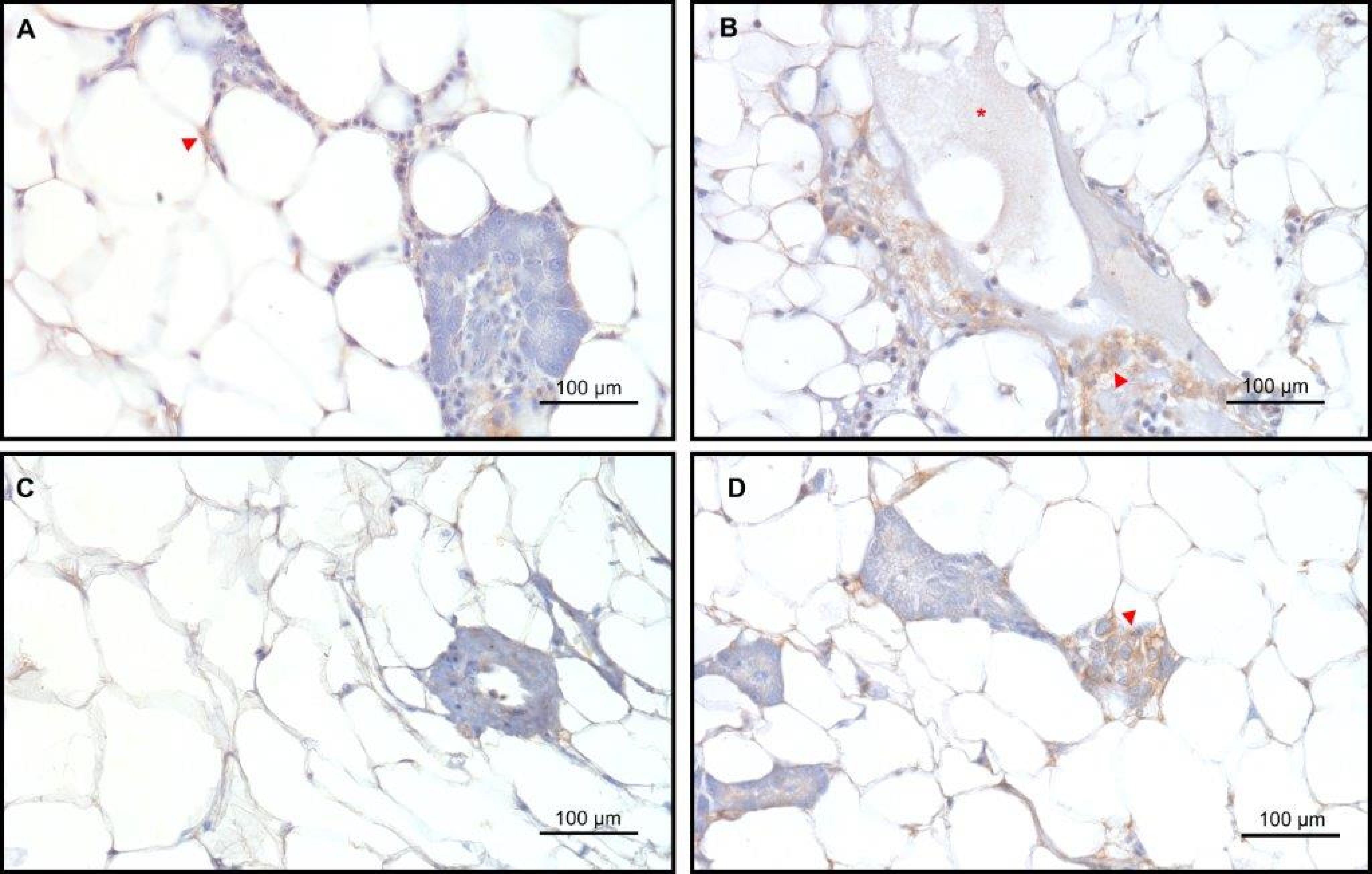
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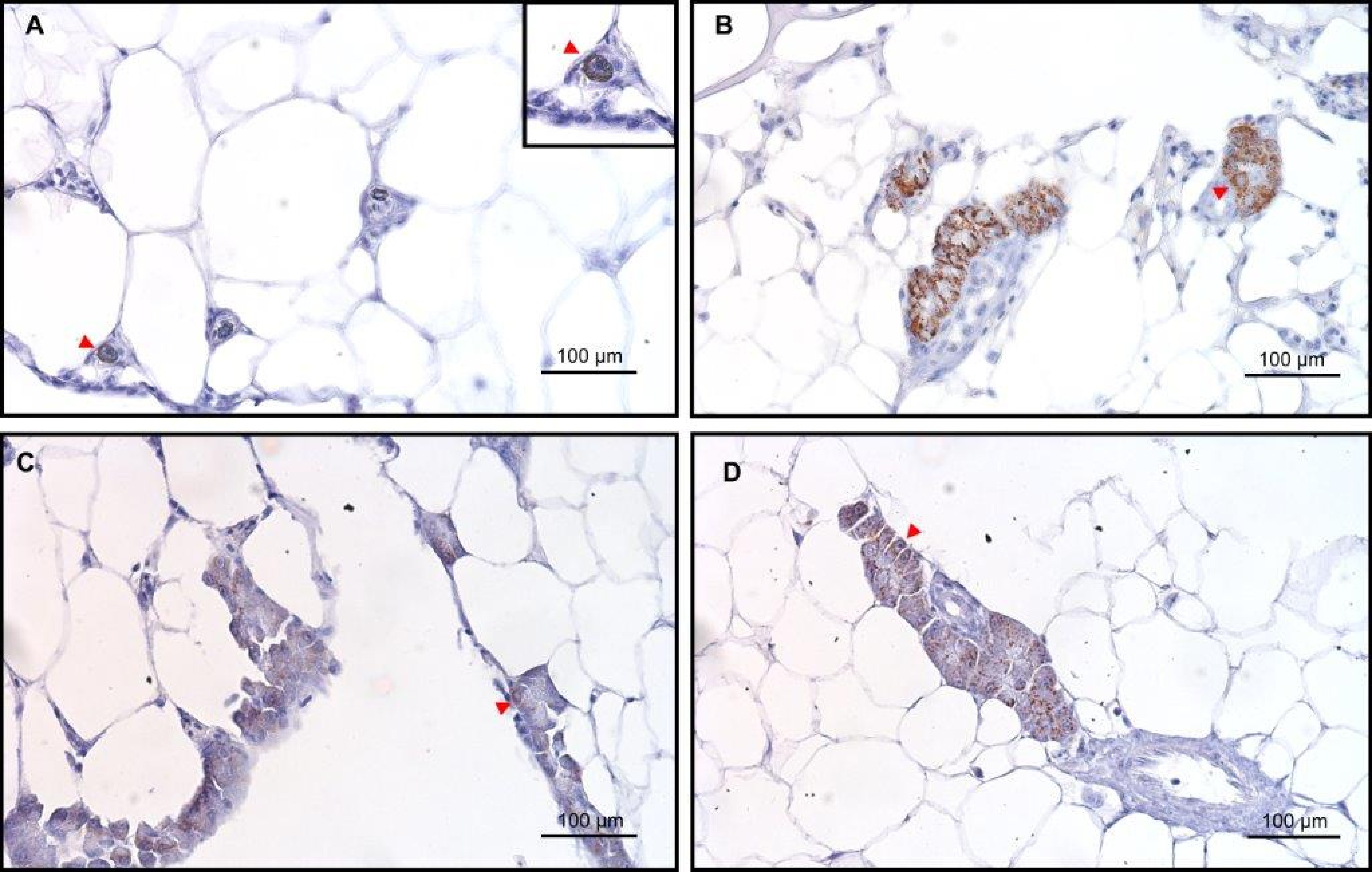
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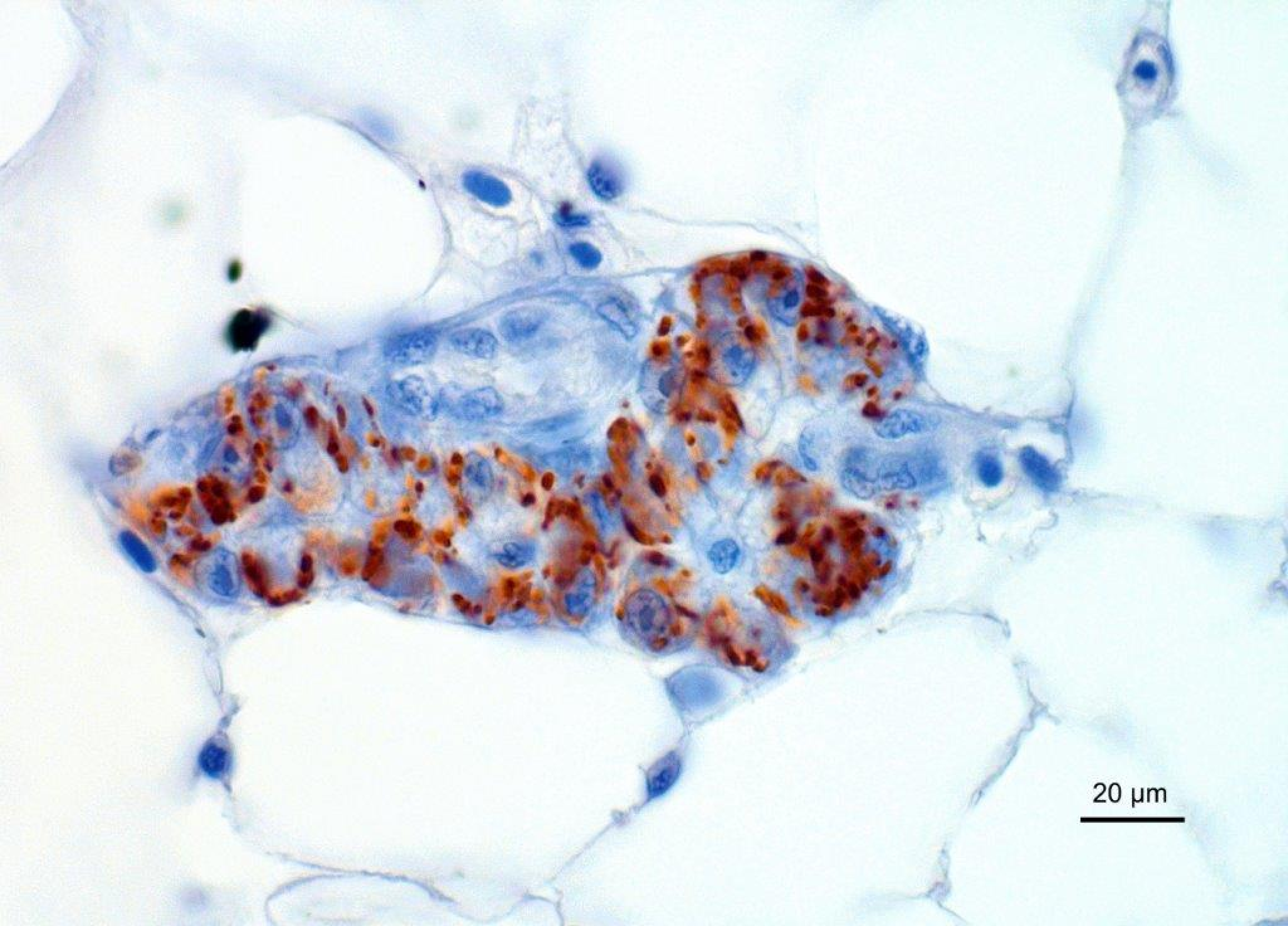
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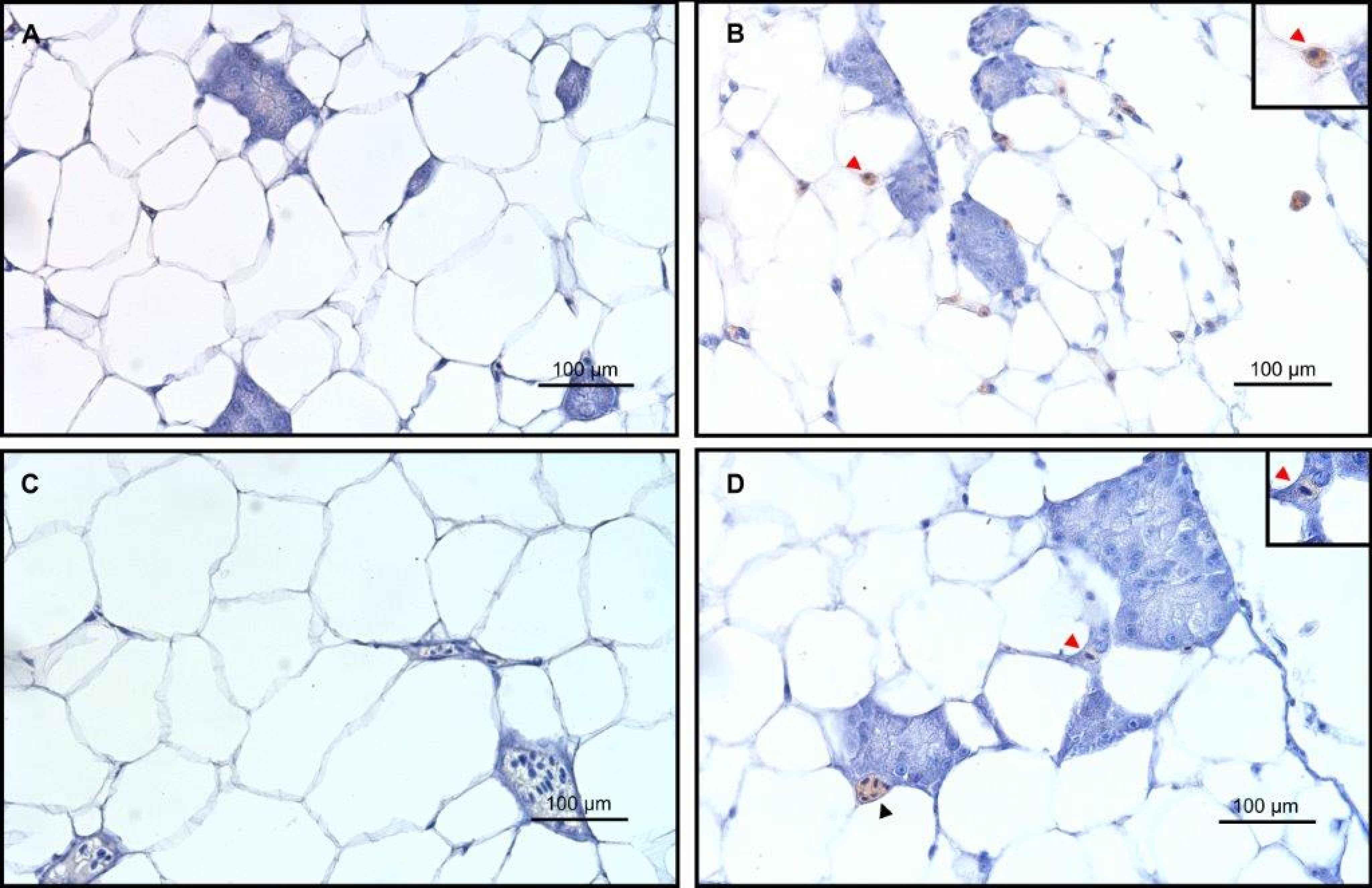
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