Insights into the development of hepatocellular fibrillar inclusions in European flounder (Platichthys flesus) from UK estuaries

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Abstract

Hepatocellular fibrillar inclusions (HFI) are an unusual pathology of unknown aetiology affecting European flounder (*Platichthys flesus*), particularly from estuaries historically impacted by pollution. This study demonstrated that the HFI prevalence range was 6-77 % at several UK estuaries, with Spearman rank correlation analysis showing a correlation between HFI prevalence and sediment concentrations of ∑PBDEs and ∑HBCDs. The data showed that males exhibit higher HFI prevalence than females, with severity being more pronounced in estuaries exhibiting higher prevalence. HFI were not age associated indicating a subacute condition. Electron microscopy confirmed that HFI were modified proliferating rough endoplasmic reticulum (RER), whilst immunohistochemistry provided evidence of VTG production in HFI of male *P. flesus*. Despite positive labelling of aberrant VTG production, we could not provide additional evidence of xenoestrogen exposure. Gene transcripts (VTG/CHR) and plasma VTG concentrations (>1 μg ml^{-1}), were only considered elevated in four male fish showing no correlation with HFI severity. Further analysis revealed that reproductively mature female *P. flesus* i.e. >3-year-old, did not exhibit HFI, whereas males of all ages were affected. This, combined with previous reports that estradiol (E2) can impair mixed function oxygenase activity, supports a hypothesis that harmful chemical metabolites (following phase 1 metabolism of their parent compounds) are potentially responsible for HFIs observed in male and ≤ 3-year-old female fish. Consequently, HFI and xenoestrogenic induced VTG production could be independent of each other resulting from different concurrent toxicopathic mechanisms, although laboratory exposures will likely be the only way to determine the true aetiology of HFI.

1. Introduction

Marine pollution has long been a subject of concern with legal and societal obligations to measure spatial and temporal trends of anthropogenic chemicals and their impact. The European Union (EU) Marine Strategy Framework Directive (MSFD) requires EU member states to demonstrate Good Environmental Status (GES) by 2020 (Directive, 2008/56/EC). This legislation largely underpins marine monitoring undertaken in the UK and EU member states. Descriptor 8 of the MSFD stipulates that chemical concentrations in the marine environment must not give rise to pollution effects. To this end, the International Council for Exploration of the Seas (ICES) Study Group on the Integrated Monitoring of Contaminants (SGIMC) developed a comprehensive monitoring approach utilising fish and invertebrate biomarkers to assess the biological effects of marine contaminants (Vethaak et al., 2017). The assessment of whole organism, tissue and sub-cellular biomarkers provide an integrating framework by which the impact of chemicals, as measured, can be assessed. This leads to increased understanding of the health status of the marine environment. The identification of specific chemical and biological interactions are particularly desirable (Lyons et al., 2010), therefore marine monitoring programmes often adopt a weight of evidence approach to investigate causal links between the presence of contaminants and biomarkers of their effects. This task is undoubtedly challenging, although previous studies have reported specific relationships between environmental chemicals and their effects, particularly in the field of hepatocarcinogenicity and endocrine disrupting toxicity (Myers et al., 1990; Waite et al., 1991; Matthiessen et al., 1995; Sumpter and Jobling, 1995; Myers et al., 1998; Allen et al., 1999b; Harries et al., 1999; Stehr et al., 2004; Raut and Angus, 2010; Chow et al., 2013).

Endocrine disrupting chemicals (EDCs) are natural or synthetic chemicals that interfere with the production and regulation of natural hormones and their subsequent effects (Damstra et al., 2002). Several aquatic EDC studies have focussed on the induction of vitellogenesis in male fish, the measurement of which serves as a biomarker of exposure to xenoestrogens (Purdom et al., 1994; Sumpter and Jobling, 1995; Folmar et al., 1996; Kime et al., 1999). Numerous studies have adopted this biomarker to investigate oestrogenic activity of chemicals in laboratory and field studies (Folmar et al., 1996; Harries et al., 1999; Folmar et al., 2001; Kleinkauf et al., 2004; Liney et al., 2005; Scott et al., 2006; Scott et al., 2007). The development of ovotestis (intersex), has previously been observed in male fish sampled from sites with high oestrogenic activity (Jobling et al., 2002). Field and

laboratory studies suggest that estuarine and marine fish species are similarly affected compared to freshwater species (Allen et al., 1999a; Allen et al., 1999b; Kleinkauf et al., 2004; Kirby et al., 2006; Scott et al., 2006; Scott et al., 2007; Velasco-Santamaria et al., 2010). These markers of exposure to marine xenoestrogens are primarily reported in estuarine species resulting from high anthropogenic inputs at these locations, although their presence has been reported sporadically in offshore species (Fossi et al., 2002; Stentiford and Feist, 2005; Scott et al., 2006; Scott et al., 2007).

Liver histopathology has been used to investigate the cause-effect relationship between environmental contaminants and the presence of toxicopathic lesions in several fish species including flatfish (Köhler, 1990; Myers et al., 1990; Vethaak and Jol, 1996; Myers et al., 1998; Stentiford et al., 2003; Stehr et al., 2004; Lang et al., 2006; Wolf and Wheeler, 2018). Hepatocellular fibrillar inclusions (HFI) are a visually striking, non-neoplastic toxicopathic lesion of unknown aetiology. Characterised by the presence of cytoplasmic "brush-like" structures of affected hepatocytes, HFI have been previously reported in the laboratory and field (Köhler, 1989, 1990; Vethaak and Wester, 1996; Stentiford et al., 2003; Lyons et al., 2004; Kuiper et al., 2007; Pal et al., 2011; Carrola et al., 2013). Moreover, Stentiford et al. (2003) showed that they are prevalent at industrialised estuaries compared to unimpacted sites sampled during the same period and show increased prevalence during autumn compared to spring. Historical Cefas data reveal that HFI are particularly prevalent (up to 80 %) in *P. flesus* from estuaries previously demonstrating high VTG plasma concentrations and ovotestis (Allen et al., 1999a; Allen et al., 1999b; Kirby et al., 2004). Ultrastructural analyses of HFI in *P. flesus* previously revealed them as proliferating rough endoplasmic reticulum (RER) and/or cytoskeletal microtubules associated with hepatocellular regeneration (Köhler, 1989, 1990). Despite previous studies, the aetiology of HFI remains unknown.

This study reports HFI prevalence in *P. flesus* from several UK estuaries sampled in 2010, incorporating pathology, chemistry (biota and sediment) and biomarker data collected as part of the UK's Clean Seas Environmental Monitoring Programme (CSEMP). We report new observations that might allude to their aetiology and warrant further investigation.

2. Materials and Methods

2.1 Field sampling

P. flesus were sampled from each estuary (n= 50), including the Alde (52.113, 1.574), Humber (53.589, -0.070), Medway (51.388, 0.521), Thames (51.504, 0.079), Tyne (54.987, -1.496) and Mersey (53.306, -2.883) estuaries during the autumn of 2010 as part of the UK's CSEMP. Fishing was conducted using a 2 m beam trawl for durations of 20 mins. *P. flesus* were transferred to aerated flow through seawater prior to sampling. Following euthanasia, blood was sampled using a heparinised syringe from the caudal vein and centrifuged at 10000 rpm for 5 mins. Plasma was snap frozen in liquid nitrogen prior to storage at -80 °C. Viscera were removed and a standardised 3-4 mm liver cross section (in addition to gonad, kidney and spleen) was obtained for formalin fixed paraffin embedded (FFPE) histology. Organs were placed into 10 % Neutral Buffered Formalin (NBF) (Pioneer Research Chemicals Ltd., UK) for 48 h prior to transferring into 70 % Industrial Denatured Alcohol (IDA) (Pioneer Research Chemicals Ltd., UK) and subsequent histological processing. Otoliths were sampled from each fish for age determination (Easey and Millner, 2008). For transmission electron microscopy (TEM) and real-time PCR (qPCR), corresponding liver samples were dissected and placed into 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) (Agar Scientific, UK) and snap frozen in liquid nitrogen, respectively. Frozen samples were stored in a dry shipper for transportation back to the laboratory and transferred to a -80 °C freezer prior to analysis. Remaining liver was pooled by sex and stored at -20 °C in *n*-hexane rinsed gas jars until chemical analysis for metals and organohalogens. Corresponding samples of muscle were obtained and stored at -20 °C for mercury (Hg) analysis. For quantification of metals, PAHs and organohalogens in sediment samples, three sediment grabs were obtained using a van Veen grab deployed at the start, middle and end of the fishing tow at each estuary. Sediments were stored in *n*-hexane rinsed 500 ml glass jars and stored at -20 °C prior to further laboratory chemical analyses. The humane killing of fish in this study was undertaken in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986.

2.2 Histology

Tissues were processed in a Leica Peloris vacuum infiltration processor using standard histological protocols and embedded in paraffin wax. Sections of 3-4 µm were obtained using a Thermo Shandon Finesse ME microtome and stained with haematoxylin and eosin (HE). Slides were examined for lesions indicative of contaminant exposure according to quality assured criteria (Feist et al., 2004) using a Nikon Eclipse Ni-U microscope. Additionally, whole liver sections were screened to estimate the proportion of hepatocytes containing HFI. A severity score was subsequently assigned to each

fish using a semi-quantitative index (Table 1). These data were later used to identify suitable samples for immunohistochemistry, TEM, and qPCR.

2.3 Immunohistochemistry (IHC)

Following histological analysis, additional tissue sections (3-5 µm) were obtained for immunohistochemical detection of VTG and microtubule α/β- tubulin. Firstly, sections were selected from representative livers (including females) exhibiting HFI, for confirmation of IHC positive labelling. Following the initial observation of VTG positive labelling of HFI in male fish using IHC, additional sections were later obtained from male *P. flesus* liver samples that were also selected for qPCR, from the Tyne (n= 28) and Mersey (n= 29). These locations yielded excellent material for further study. Briefly, sections were dewaxed and rehydrated prior to heat-induced epitope retrieval (HIER) using a pressure cooker containing 50 mM sodium citrate buffer. Once maximum pressure was achieved, sections were incubated for 10 minutes prior to removal from pressure cooker, cooling and washing in distilled water. Endogenous biotin activity was blocked using 0.05 % avidin and 0.05 % biotin in tris buffered saline (TBS) with intermediate and final washing steps in TBS. Sections were subsequently treated using a modified protocol of the Leica Immunohistochemistry Peroxidase Detection System (RE7110, Leica, UK) incorporating a (1) polyclonal anti-rabbit flounder VTG primary antibody (Allen et al., 1999b) at 1:5000 dilution or (2) a commercially available α/β- tubulin polyclonal primary antibody for zebrafish (*Danio rerio*) (#2148, Cell Signaling Technology, USA) (1:50). Negative controls were achieved by substituting primary antibody for TBS.

2.4 Transmission electron microscopy (TEM)

Selected samples corresponding to *P. flesus* exhibiting HFI were processed for TEM. Following fixation, samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed for 1 h in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were washed in three changes of 0.1 M sodium cacodylate buffer prior to dehydration by graded acetone series. Samples were embedded in Agar 100 epoxy (Agar Scientific, UK) and polymerised at 60°C overnight. Semi-thin sections (1-2 µm) were obtained using a Leica EMUC7 ultramicrotome, stained with toluidine blue and examined for areas of interest. Targeted areas were sectioned further to produce ultra-thin sections (70-90 nm). Sections were mounted on uncoated copper grids and stained with 2 % aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1210 transmission

electron microscope with images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.5 Real-time polymerase chain reaction (qPCR)

Vitellogenin (VTG) and choriogenin (CHR) gene transcripts (Supplementary Table 1) were quantified from representative samples of male *P. flesus* liver obtained from fish collected at the Tyne and Mersey estuaries. This aspect was conducted to investigate the relationship between gene expression and HFI Severity (not for the comparison of gene expression between sampling locations). The Tyne and Mersey were chosen because they yielded high quality histological material for further analyses. Total RNA was extracted from up to 40 mg frozen liver samples using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, UK) following manufacturers protocols and quantified using a Nanodrop (Applied Biosystems, UK). Reverse transcription was done in a 20 µl reaction following Superscript II protocol using 1 µl SuperScript II; oligo dT primers (0.25 µl) (Invitrogen, Life Technologies, UK); random primers (0.6 µl) (Promega, UK); 1 µg of total RNA; and nuclease free water (Fisher Scientific, UK). Reaction mixes were kept cool on ice prior to incubation at 25 °C, 50 °C and 70 °C for 5 minutes, 60 minutes and 15 minutes respectively. Resulting cDNA was diluted 20-fold prior to use. Quantification of gene transcription was undertaken by qPCR. Each reaction was done in duplicate using an Applied Biosystems Step-One Plus qPCR system, with transcription normalised to ubiquitin (UBQ), elongation factor 1 (EF1), F-actin (FACT) and α-tubulin (ATUB) housekeeping genes (Supplementary Table 1). Each PCR comprised: 10 µl of Promega GoTaq® qPCR Master Mix (Promega, UK), 0.2 µM of each relevant primer, 4 µl of diluted cDNA and nuclease free water to a total volume of 20 µl. Cycling of 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 seconds, and 60 °C for 1 min. Melt curve analysis was done at 95-60 °C with fluorescence recorded every 0.3 °C. Data was exported to the LinRegPCR analysis software for determination of baseline fluorescence, PCR efficiency and Cq value (Ruijter et al., 2009). Subsequent analysis was undertaken using Relative Expression Software Tool (REST) 2009 (Corbett Research Pty Ltd, Germany) for relative gene expression analysis (Pfaffl et al., 2002). Comparison of *P. flesus* expression data was conducted between biological groups comprised of HFI severity stage.

2.6 VTG analysis

VTG analysis was performed using a homologous, enzyme-linked immunosorbent assay (ELISA) (Kirby et al., 2004). VTG concentrations were determined to investigate the extent of vitellogenesis in male *P. flesus* sampled from all estuaries.

2.7 Metals analysis (sediment)

Analysis was carried out using total sediment digestion (with hydrofluoric acid) on the fine sediment fraction (<63 µm) by means of methods described by Jones and Laslett (1994). Quantification of metals (Cr, Ni, Cu, Zn, As, Cd, Pb, Hg, Mn, V, Al, Fe, Li, Rb) was conducted using inductively-coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma-atomic emission spectrometry (ICP-AES) as detailed in Lyons *et al*., (2015).

2.8 Polycyclic aromatic hydrocarbons analysis (sediment)

Quantification of 34 parent polycyclic aromatic hydrocarbons (PAHs) and groups of alkylated PAHs was performed using coupled gas chromatography-mass spectrometry (GC-MS) in electron impact ionization mode as detailed in Lyons *et al.,* (2015a). The limit of detection in sediment was set at 0.1 µg kg^{-1} dry weight for each PAH compound or group.

2.9 Organohalogen analysis (sediment)

A total of 11 polybrominated diphenyl ether (PBDE) congeners and the 3 diastereoisomers α-, β-and γ- hexabromocyclododecane were analysed by methods as detailed in Lyons *et al.*, (2015a). All data were normalised to total organic carbon present (<2 mm sediment fraction).

2.10 Metals analysis (biota)

Analysis was carried out using acid microwave digestion of liver samples (Jones and Laslett, 1994). Quantification of Cd and Pb in liver tissue, and Hg in muscle was performed by inductively coupled plasma-mass spectrometry (ICP-MS) and inductively-coupled plasma-atomic emission spectrometry (ICP-AES) as detailed in Al-Zaidan *et al.* (2015).

2.11 Organohalogen analysis (biota)

A total of 25 and 11 polychlorinated biphenyl (PCB) and PBDE congeners respectively were analysed by GC-ECD and GC-MS, respectively, according to analytical methods as detailed in Al-Zaidan *et al.* (2015) and Lyons *et al.*, (2015b). All data were normalised to percentage lipid content.

252

253 *2.12 Data analysis*

254 Chi-square test, Mann-Whitney rank sum test, Spearman's rank correlation and linear regression
255 analysis were undertaken in SigmaPlot version 12.0, from Systat Software, Inc. (San Jose California
256 USA). Kruskal-Wallis rank sum test was undertaken in R v.2.7.0 (R Development Core Team, 2008).

257

258 **3. Results**

259

260 *3.1 Histology*

261 Histological analysis revealed the presence of HFI in the liver of *P. flesus* collected from all sites at
262 varying severity and prevalence (Figure 1). Affected hepatocytes contained fine "brush-like" filaments
263 arranged into arrays and exhibited cellular atrophy. Closer analysis revealed basophilic filaments in
264 HE sections (Figure 1b) and were observed at varying degrees of cytoplasmic coverage. Some
265 hepatocytes possessed relatively few filaments whereas severely affected cells exhibited compact
266 filaments occupying most of the cytoplasm. Transverse HFI sections appeared as clusters of
267 individual basophilic spots. HFI were sometimes displaced towards the periphery of hepatocytes
268 possessing increased lipid content. HFI were observed in either relatively few cells scattered
269 throughout the liver; in discrete patches of hepatocytes; or throughout much of the liver in severely
270 affected fish. A small number of fish possessed what appeared to be degenerative HFI, characterised
271 by distorted and atrophied inclusions, although this was inconclusive. A further observation within
272 many affected hepatocytes was the presence of eosinophilic deposits situated within the cytoplasm
273 (Figures 1c and 1d). This material resembled a substance previously confirmed as VTG (Folmar et al.,
274 2001) and was observed in both male and female fish.

275

276 A varying prevalence and severity of HFI was identified at all sampling sites, with the Mersey and the
277 Alde being the worst and least affected sites respectively. Male and female fish were differentially
278 affected (Figures 2a and 2b) with male *P. flesus* exhibiting 30 % prevalence compared to 11.7% in
279 females across all sampling locations (Chi-square test, p <0.001). The following data in parenthesis
280 indicate the HFI percentage prevalence for all fish, male fish and female respectively: Mersey (77.0,
281 79.3, and 57.1), Tyne (60.0, 78.6, and 36.4), Thames (46.0, 63.6, and 32.1), Medway (36.0, 44.7, and
282 8.3), Humber (26.0, 34.5 and 14.3), and Alde (6.0, 3.7, and 8.7). Spearman Rank Order Correlation
283 revealed a perfect positive correlation between prevalence and mean HFI severity stage in all fish

(males and females combined) at the six estuaries sampled (r_s= 1.0, p = 0.002, n= 6). Livers sampled from the Mersey and Tyne estuaries yielded a good frequency of the numerous HFI severity stages. As a result, male fish from these estuaries were selected for further immunohistochemical, ultrastructural and molecular analysis.

3.2 Age determination

Length and weight ranges exhibited considerable overlap between individual ages (Supplementary Table 2). Most *P. flesus* sampled were between 2-4 yrs of age (Figure 3). Analysis of age and HFI from male *fish* revealed that the prevalence range of HFI was 46-67 % for ages 1-7 yrs (Figure 3a). The prevalence was 100 % for 8-year-old fish, although this was based on two individuals. Indeed, ages 5-8 generally possessed fewer numbers of fish (Figure 3a). The mean age for all male *fish* with and without HFI was 3.3 and 3.0 respectively indicating no association between age and prevalence (Mann-Whitney Rank Sum Test, p=0.571). Statistical analysis revealed an interaction between age and female *P. flesus* with HFI (Mann-Whitney Rank Sum Test, p=0.002)- no female fish over the age of 3 years were affected, although ages 1-3 yrs all had a prevalence of ≈35 % (Figure 3b).

3.3 Immunohistochemistry (IHC)

Immunohistochemistry was undertaken using a homologous polyclonal anti-rabbit *P. flesus* VTG primary antibody. Negative controls had no background diaminobenzidine staining (Figure 4a). IHC demonstrated that VTG readily associated with HFI. Labelling was specific with minimal non-specific background staining (Figures 4b-4d). Previously identified eosinophilic deposits in HE sections (Figures 1c and 1d) corresponded to a substance demonstrating positive VTG labelling (Figure 4c inset). Comparisons between HE and IHC sections showed all HFI labelled VTG positive. A commercially available α/β- tubulin polyclonal antibody was used to confirm previous observations of microtubules within the RER cisternae lumina (Köhler, 1989). Although the antibody claimed cross reactivity for all species (Cell Signalling Technology, α/β-Tubulin Antibody #2148, New England Biolabs, UK), IHC demonstrated negative labelling for α/β- tubulin within *P. flesus* tissues tested.

3.4 Transmission electron microscopy (TEM)

Normal hepatocytes possessed rounded nuclei with well-defined nucleoli, typically exhibiting no cellular atrophy. Mitochondria, Golgi complex and lysosomes were clearly visible (Figure 5a). Nuclei were surrounded by RER cisternae with associated ribosomes at the outer membrane. Affected

hepatocytes demonstrated a distended cytoplasm and polygonal appearance. The HFI appeared as arrays orientated across the longest hepatocellular axis (Figure 5b-5d). Close inspection revealed ribosomes (28-32 nm) located along their entire length (Figure 5e) confirming that HFI were significantly modified RER cisternae. HFI were interspersed with mitochondria and occasional non-membrane bound lipid like inclusions (Figure 5b and 5c). HFI in female livers were frequently electron dense in appearance, apparently caused by increased numbers of free ribosomes interspersed between cisternae. These hepatocytes always contained a dense pleomorphic nucleus (2-3 µm) with poorly defined nucleolus and irregular border. Hepatocytes containing HFI in male fish contained a nucleus that was similar in size (5-6 µm) to normal hepatocytes. The HFI occasionally exhibited some fragmentation (Figure 5f), which was not the result of poor fixation, evidenced by the presence of mitochondria with well-defined cristae. Autophagosomes were observed containing degenerate membranous substance suggesting autophagy in affected hepatocytes. This material was frequently seen within bile canaliculi. Autophagosomes were often seen undergoing coalescence with (or immediately associated with) lysosomes (Figure 5d). Similarly, lysosomes occasionally appeared to demonstrate coalescence with non-membrane bound lipid-like substance (Figure 5c and 5f). This appeared to correspond to the eosinophilic substance observed in HE sections (Figure 1c and 1d).

3.5 Real-time polymerase chain reaction (qPCR)

The reaction efficiency for qPCR of VTG, CHR, UBQ, EF1, FACT and ATUB was 1.826, 1.822, 1.963, 1.850, 1.940 and 1.790 respectively. Analyses of gene transcripts was carried out for VTG and CHR in liver of male *P. flesus* sampled from the Mersey and Tyne estuaries. Linear regression analysis between VTG and CHR transcripts showed a strong positive linear correlation (r= 0.981). Relative quantities of VTG and CHR gene transcripts were similar across biological groups comprised of HFI severity stages (Supplementary Figure 1). Kruskal-Wallis rank sum test revealed no association between relative quantities of gene transcripts and biological groups (p= 0.3098 and p=0.5317 respectively). The lowest and highest levels of VTG gene transcription in individual fish differed by over 8000-fold.

3.6 Analysis of plasma vitellogenin (VTG)

The ELISA determined VTG concentrations in 171 plasma samples of male *P. flesus* (Table 2). VTG concentrations were low for nearly all *P. flesus* sampled. Ten fish exhibited VTG concentrations >1 µg ml^{-1} (range 1.7-1944.0 µg ml^{-1}) across all sites, with remaining fish demonstrating concentrations

similar to baseline levels observed during previous monitoring programmes (Kirby et al., 2004). Four fish exhibited relatively high VTG concentrations from the Mersey (8.7 and 672.9 µg ml^{-1}) and Tyne (897.9 and 1944.0 µg ml^{-1}). Overall, mean plasma VTG concentrations were ranked as follows: Tyne > Mersey > Alde ≈ Humber ≈ Medway ≈ Thames. No direct relationships were observed between HFI, gene transcripts and VTG concentrations in analysed fish from i.e. Tyne and Mersey (Supplementary Figure 2).

3.7 Chemistry (biota and sediment)

Concentrations of contaminants measured in pooled liver and sediment samples are presented in supplementary tables 3-8. A Spearman's rank correlation test was used to determine potential relationships between the prevalence of HFI in male fish (arcsine transformed data) and the concentration of contaminants. Differences were seen between individual metals and between sites, although no relationship was observed between metal concentrations and HFI prevalence. Summary data for ∑PBDE, ∑HBCD, ∑PCB and ∑PAH is presented in Table 3. Analysis of PAHs showed that the range of THC concentration was 43.1-2857.7 mg kg^{-1} d.w. (Mersey and Tyne respectively), whilst ∑PAH concentrations ranged from 316.6 – 34850.9 µg kg^{-1} d.w. (Mersey and Medway respectively). Spearman rank correlation analysis revealed a moderate positive correlation (r_s= 0.90, p = 0.083, n= 5) between increasing THC concentration and HFI prevalence, although this was insignificant (see discussion). Analysis of PBDEs and HBCDs in sediment indicated that BDE#209, BDE#99, BDE#47 congeners and ∑HBCD isomers represented the largest proportion measured between estuaries, with BDE#209 contributing ≈75-95 % of all flame retardants measured (range 266.67-11,885.93 µg kg^{-1} d.w.). Following Spearman rank correlation analysis, ∑PBDEs, BDE#209 and ∑HBCDs each showed a strong positive relationship with HFI prevalence (r_s= 0.886, p = <0.033, n= 6).

The ∑PBDEs in pooled *P. flesus* liver samples ranged from 49.09–679.63 µg kg^{-1} l.w., whilst ∑HBCD ranged from 4.69–298.83 µg kg^{-1} l.w. (Table 3). Concentrations were lowest and highest at the Alde and Thames, respectively. Congeners BDE#47, BDE#100 and ∑HBCD isomers represented the highest concentrations measured in liver, with BDE#47 contributing ≈40-55 % of all flame retardants measured at all locations (range 55.25-491.66 µg kg^{-1} l.w.). No significant relationship was observed between ∑PBDEs and ∑HBCD concentrations in liver and HFIs in males. The ∑PCBs concentrations in pooled liver samples of male *P. flesus* ranged from 322.48-4647.99 µg kg^{-1} l.w. at Alde and Mersey respectively. Spearman rank correlation analysis demonstrated a moderate positive correlation

between ∑PCBs in liver and HFIs in males, although it should be noted that this relationship bordered on the threshold of significance (r_s= 0.829, p = 0.058, n= 6).

4. Discussion

This study combined available data collected as part of a routine estuarine monitoring programme with further laboratory analyses to help improve our understanding of the development of HFI in *P. flesus*. Previous studies have highlighted age as a fundamental factor for distinguishing acute and chronic diseases (Vethaak et al., 1992; Vethaak and Jol, 1996; Stentiford et al., 2010). Our data demonstrated the presence of sexual dimorphism concerning the relationship between age and HFI prevalence. Although 2-4-year-old fish were the most frequent ages sampled, all ages of male *P. flesus* contained approximately equal proportions of affected and unaffected fish (Figure 3a). Interestingly, we observed that female *P. flesus* over 3 years old did not exhibit HFI (Figure 3b); the age at which sexual maturity is ordinarily reached (Summers, 1979). Whilst fewer numbers of female *P. flesus* were present in older age classes (Figure 3b), our observation is substantiated by the presence of sufficient numbers of unaffected 4-year-old female fish (n= 20). Carrola *et al.* (2013) used fish length as a surrogate for age and showed that the smallest and largest *P. flesus* sampled from the Douro estuary, Portugal, exhibited a significantly lower HFI prevalence compared to those of intermediate length. However, length is not necessarily reliable for this purpose and varies considerably between regions (Stentiford et al., 2010). Extrapolation of Douro *P. flesus* lengths to our data show that the smallest and largest Douro fish were potentially anywhere between 1 and 6 yrs old (Carrola et al., 2013). Consequently, it was impossible to deduce the age of Douro *P. flesus* for comparisons to our own data. Based on our observation that all ages of male *P. flesus* contained approximately equal proportions of affected and unaffected fish, and the previously reported seasonal differences (Stentiford et al., 2003), we conclude that HFI are a subacute response affecting male and sexually immature female *P. flesus*.

In this study, ultrastructural analysis confirmed previous reports that HFI are formed from proliferating RER exhibiting extensive disorganisation. Köhler (1989; 1990) described the presence of enlarged microtubules (macrotubules) within RER cisternae lumina of *P. flesus* following pre-treatment with Tannic acid (Mizuhira and Futaesaku, 1971; Köhler, 1990). It was proposed that macrotubules resulted from the incorporation of tubulin subunits from the cytoplasmic pool into RER cisternae, thus

causing HFI. IHC using an α/β- tubulin polyclonal primary antibody did not confirm their presence despite confirmed antibody species reactivity for zebrafish and highly conserved tubulin genes between species (Wade, 2007). Ordinarily, it is perhaps unclear how tubulin subunits are incorporated into the RER for the formation of macrotubules (unpublished data, see Köehler, 2004). Free ribosomes are primarily responsible for the synthesis of proteins destined elsewhere in the cytoplasm. Proteins that are synthesised on ER bound ribosomes primarily pass directly through the cisternae membrane into the RER lumen where they are packaged into vesicles, released from the RER and transported to various cytoplasmic locations, via the Golgi complex, such as the lumen of other organelles or the plasma membrane. In our study, putative macrotubules were only observed by TEM in a single hepatocyte from one fish. However, this was inconclusive since our samples were not pre-treated with tannic acid, therefore this observation was likely caused by the angle of section. The role of tubulin in the formation of HFI in our study remains inconclusive.

P. flesus primarily reside within estuaries and migrate annually to open ocean spawning grounds during the winter once sexual maturity is reached (Summers, 1979). Spawning typically occurs between February and May, after which *P. flesus* return inshore to feed during the summer. Concentrations of contaminants are generally higher in UK estuaries compared to the open sea (Woodhead et al., 1999). Two possible hypotheses are framed by these observations: (1) HFI result from natural seasonal factors specific to estuarine habitation, or (2) HFI result from seasonal exposure to anthropogenic contaminants. These links between HFI and the environment assume that fish sampled during autumn-winter have spent several months within an estuary. Observations of seasonal differences in HFI prevalence (Stentiford et al., 2003) and the migratory behaviour of *P. flesus, also* support this theory. This study compared the prevalence of HFI in male fish to chemical concentrations measured in corresponding sediment and biota samples. Whilst numerous chemical data were available, investigations into the relationship between contaminants and HFI were difficult since (1) liver samples were pooled to obtain enough tissue for chemical analysis, and (2) only 6 data points per chemical (corresponding to each estuary), were available. These factors were unavoidable due to the nature of chemical analyses and the biological end point i.e. HFI prevalence. Despite these limitations, a simple correlation analysis was attempted to provide insight into potential relationships between contaminants and HFIs. No correlations were observed with concentrations of individual PBDE congeners or ΣPBDEs in biota, although correlations were made with ΣPCBs. Our data demonstrated a strong positive correlation between HFI prevalence and sediment concentrations of

ΣPBDEs and ΣHBCDs. The relationship with ΣPBDEs was largely influenced by the relatively large proportion of BDE#209 detected at all estuaries compared to other congeners. Previously, BDE#209 was not considered to be a major risk to aquatic organisms, primarily due to its hydrophobicity, high molecular weight and reduced biological uptake. However, studies have shown that BDE#209 undergoes biotransformation into more persistent lower brominated congeners with increased toxicity (Birnbaum and Staskal, 2004; Stapleton et al., 2006; Munschy et al., 2011). Observations of a positive correlation between ΣHBCDs in sediments and HFI prevalence is curious. HFI were previously reported in 83 % of *P. flesus* following chronic exposure to environmentally relevant concentrations of HBCD (Kuiper et al., 2007). However, despite using several concentrations, including higher concentrations than those measured in our study, no dose dependant response was observed during that study. Whilst no significant relationship was observed with ΣPAHs, it is worth noting that THC concentration at the Mersey was significantly lower than anticipated. This was largely explained by the Mersey sediment substrate sampled being relatively sandy in comparison to sediment samples obtained elsewhere i.e. mud. Removal of the Mersey outlier resulted in a positive correlation (r_s= 0.90), although this removal of a data i.e. n= 5, resulted in an insignificant correlation (p = 0.083).

Despite these observations, we should consider potential species-specific biological factors that may influence HFI development. Ultrastructural changes in hepatocytes were reported in winter flounder (*Pleuronectes americanus*) associated with the synthesis of an antifreeze protein (March and Reisman, 1995). Whilst HFI were not the subject of that study, it highlights a unique liver function in that species. Since HFI are more commonly observed during autumn/winter in *P. flesus* (Stentiford et al., 2003), one might consider similar biological functions concerning migrations from brackish to marine environments (anticipating changes in salinity and/or temperature) as a possible cause. However, HFI have been observed, albeit rarely, in the wholly marine flatfish *Limanda limanda* (Cefas, unpublished data) suggesting this is not the case. Furthermore, this study revealed a differential prevalence between estuaries during the same sampling period and a rudimentary relationship between PBDEs and HBCDs (sediment), and PCBs (biota). The identification of relationships between specific contaminants and biological effects in the marine environment is notoriously difficult due to the presence of complex chemical mixtures and effects that potentially exist. Whilst we were unable to determine a definitive causal link, our observations as well as previous studies, support the hypothesis that chemical contaminants appear to play a role in HFI

development. Since levels of HBCDs, PAHs, and PBDEs often correlated with HFI prevalence, they present themselves as potential candidates for future study.

The PBDEs have previously been shown to elicit *in vitro* oestrogenic effects in fish hepatocytes (Nakari and Pessala, 2005; Søfteland et al., 2011). Nakari and Passala (2005) reported a clear dose dependant relationship between exposure to BDE#47, BDE#99, BDE#153 and BDE#205, and VTG synthesis and secretion. Søfteland et al., (2011) reported a significant up-regulation of hepatic ER-responsive genes (VTG and ZP3) following exposure to BDE#47 and a chemical mixture of BDE#47, BDE#99, BDE#153 and BDE#154. In our study, positive IHC labelling of VTG with an immediate association with HFI and a hepatic cytoplasmic substance, lead us to believe that HFI may result from stimulation of oestrogen receptors to produce VTG. Direct comparisons between HE and corresponding IHC sections confirmed this substance was VTG. Similar eosinophilic substances were observed in hepatocytes of fish exposed to oestrogenic compounds (Wester and Canton, 1986; Folmar et al., 2001; Zaroogian et al., 2001). Proteins, such as VTG, that are destined for use by tissues elsewhere, utilise the co-translational translocation pathway and are ordinarily synthesised on ER bound ribosomes passing through the cisternae membrane and into the RER lumen (Wolfe, 1993). Zaroogian *et al.* (2001) proposed that the occurrence of VTG within the cytoplasm of male *P. dentatus* hepatocytes resulted from the absence of oocytes in which to sequester VTG from the blood. It was proposed that glomerular damage triggered reabsorbtion of VTG back into the circulatory system followed by transportation to the liver and accumulation in lysosomes, where it is structurally broken down. Close examination of TEM sections in our study revealed that hepatocellular VTG accumulations were not membrane bound, indicating that reabsorbtion via endocytosis into the lysosomal compartment was unlikely. This indicated that VTG accumulated in hepatocytes immediately following synthesis, perhaps resulting from perturbations in the co-translational translocation pathway.

Following positive immunohistochemical detection of VTG associated with HFI, we investigated whether corresponding mRNA transcription and VTG protein translation was evident. This was achieved using qPCR and ELISA for the detection of hepatic VTG/CHR gene transcripts and plasma VTG protein respectively, in male *P. flesus*. Fish were sampled during the autumn, therefore were likely to have resided in the estuaries for several months prior to offshore migrations. If biologically relevant concentrations of xenoestrogens were present in the estuaries, *P. flesus* whould have

507 received prior exposure to them. Our results revealed no significant differences concerning VTG/CHR
508 transcription levels between Mersey and Tyne *P. flesus* exhibiting different HFI severity stages
509 (Supplementary Figure 1). Activation of VTG/CHR genes and transcription could have occurred prior
510 to the formation of HFI. The relatively short VTG mRNA half-life of 3 days (Craft et al., 2004) and the
511 significant temporal changes of transcription over a period of continuous exposure could have been
512 responsible for this. However, the ELISA results also demonstrated low concentrations of plasma
513 VTG in most male fish. The relatively high mean plasma VTG concentrations of 23.7 and 101.8 μg ml^{-1}
514 for the Mersey and Tyne respectively, result from two fish at both estuaries exhibiting high
515 concentrations of VTG (8.7 and 672.9 μg ml^{-1}; 897.9 and 1944.0 μg ml^{-1} respectively). These fish also
516 demonstrated high VTG transcription levels. However, no direct relationships were observed between
517 (a) gene transcripts (b) HFI and (c) VTG concentrations in these four individuals (Supplementary
518 Figure 2).
519
520 In our study, the majority of VTG plasma concentrations in male *P. flesus* from all estuaries were
521 similar to baseline levels observed in *P. flesus* from the Alde estuary during previous studies. This
522 observation is consistent with Kirby *et al.* (2004) who reported decreasing male flounder plasma VTG
523 concentrations from the Mersey (19,226.2 and 3.5 μg ml^{-1}) and Tyne (448.3, and 0.5 μg ml^{-1}) between
524 1996 and 2001, respectively. This observation, accompanied by the comparatively high VTG protein
525 half-life of 13-15 days (Allen et al., 1999b; Craft et al., 2004), confirms that previously impacted UK
526 estuaries have significantly improved regarding concentrations of xenoestrogens. Curiously, despite
527 the relatively low VTG protein levels as measured by ELISA in our study, IHC demonstrated clearly
528 defined VTG labelling of HFI leaving little doubt that (a) labelling was highly specific and (b) VTG was
529 being synthesised in hepatocytes despite the lack of significant concentrations measured in the
530 plasma. A potential explanation for this observation is that VTG concentrations in plasma were below
531 the ELISA limit of detection (0.2 μg ml^{-1}), although still detectable within hepatocytes using IHC.
532 Alternatively, the discrepancy may be related to additional mechanisms of toxicity affecting the co-
533 translational translocation pathway e.g. protein mis-folding, preventing extracellular secretion,
534 allowing the detection within the site of production (liver) but not in the site of transport (blood). This
535 is supported by observations of non-membrane bound hepatocellular VTG accumulations and
536 accompanying autophagy, which is responsible for the complete degradation of aggregated proteins
537 (Mandl et al., 2013).
538

539 Histological markers of endocrine disruption in the aquatic environment have primarily been confined
540 to observations of ovotestis in the gonads of male fish (Allen et al., 1999b; Harries et al., 1999;
541 Jobling et al., 2002; Stentiford and Feist, 2005; Tyler and Jobling, 2008; Bizarro et al., 2013). Whilst
542 we initially perceived that our observation of VTG-positive IHC labelling implicated VTG production in
543 HFI formation, their development and VTG production may be independent of each other, resulting
544 from different concurrent toxicopathic mechanisms. This is completely plausible since estuaries
545 significantly impacted by xenoestrogens likely contain other classes of contaminants. Previously
546 published evidence also suggests that this may indeed be the case. Perhaps one of the most
547 fundamental observations in our study was that female *P. flesus* >3 years (the age that female
548 become reproductively mature) did not exhibit HFIs. Kirby et al (2007) demonstrated that EROD
549 activity (a measure of mixed function oxygenase activity enabling animals to oxidise contaminants
550 including PAHs and dioxin) was suppressed in male *P. flesus* following laboratory exposure to the
551 female reproductive hormone estradiol (E2). In that study, the concentration of E2 required to
552 suppress EROD activity in males exposed to a PAH was nearly two orders of magnitude less than the
553 concentration of E2 required to induce VTG production. Similar effects were also reported in Atlantic
554 salmon (Aruke et al., 1997). This suggests that mature female *P. flesus* (i.e. those actively
555 synthesising E2) displayed reduced oxidation of PAHs and dioxins that might be encountered. This
556 hypothesis is supported by the report that, during a field survey of liver EROD activity in UK estuaries,
557 it was demonstrated that where statistically significant differences existed between males and
558 females, it was female *P. flesus* that exhibited lower EROD activity (Kirby et al., 2004). One
559 consequence of Phase I metabolism of some PAHs and dioxin is the formation of metabolites that far
560 greater toxicity than the parent compounds. Therefore, we tentatively suggest (and we stress it is
561 only a hypothesis) that one or more of these toxic metabolites might be implicated in the formation of
562 HFIs, and the reason that HFIs are not present in reproductive females is because their formation by
563 Phase I metabolism has been suppressed, most likely as a consequence of E2 production in the
564 ovaries.
565
566 The complexities and limitations of studying field samples means we cannot rule out that VTG
567 production is in some way implicated. The presence of HFI and positive IHC VTG labelling always
568 occurred together so this could be a worthwhile avenue of investigation. The next logical step to
569 identify a causal link would be to undertake laboratory exposures, using contaminants known to cause
570 oestrogenic effects and hepatic toxicity (and in combination), perhaps incorporating those

571 contaminants that exhibited rudimentary correlations in this study. It is likely that only through
572 laboratory exposures will we identify the aetiology of HFI.
573
574 **5. Conclusions**
575
576 This study reports that HFI were prevalent in *P. flesus* sampled from several UK estuaries during
577 2010 and were confirmed as a significant proliferation and disorganisation of the RER. The
578 observation that approximately equal proportions of male *P. flesus* of all ages are affected indicate
579 that HFI are a subacute pathological condition. The differential prevalence between several UK
580 estuaries of varying contaminant burdens during the same sampling period, suggest an
581 anthropogenic aetiology, although this was challenging to elucidate. The HFI prevalence appeared to
582 correlate to sediment concentrations of ∑PBDEs (largely influenced by BDE#209) and ∑HBCDs. Our
583 analysis of gene transcripts and VTG blood plasma concentrations did not provide significant
584 evidence to support the hypothesis that HFI development have a purely oestrogenic aetiology.
585 Furthermore, we showed that whilst HFI consistently exhibited positive IHC labelling for VTG, our
586 observation that female *P. flesus* over 3 years old did not exhibit HFI, and that E2 can reportedly
587 reduce mixed function oxygenase activity, it is possible that compounds other than xenoestrogens
588 could be implicated during HFI development.
589
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Table 1-3

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Stage	Type	Description
0	Absent	No hepatocytes affected.
1	Present	Individual cells containing HFI scattered within parenchyma, occupying ≤25 % of whole liver section. HFI are only present in very few fields of view (FOV) i.e. some FOV may not contain HFI. HFI mostly observed within small/condensed hepatocytes.
2	Elevated	25-50 % of hepatocytes contain HFI, although may only occupy 10-25 % of a FOV in some cases i.e. HFI may not be evenly distributed between each FOV. Affected hepatocytes appear as individual scattered cells interspersed with unaffected hepatocytes.
3	Intermediate	50-75 % of hepatocytes contain HFI appearing as both small/condensed and enlarged with many fibrils. In some areas of the liver, there is a marked increase in the frequency of affected hepatocytes resulting in the occurrence of discrete regions comprised of affected hepatocytes immediately adjacent to each other. All fields of view contain hepatocytes exhibiting HFI.
4	Abundant	≥75 % of hepatocytes contain HFI. Affected hepatocytes appear enlarged with many condensed fibrils. The increased frequency of HFI results in large regions exhibiting affected hepatocytes that are immediately adjacent to each other. Some regions may still exhibit scattered HFI interspersed with unaffected cells.
5	Degenerative/ unknown	Hepatocytes contain atrophied cytoplasmic material of unknown origin, appearing degenerative in nature. Uncertain if related to HFI.

Table 1: Semi-quantitative scoring criteria developed for grading HFI severity in whole liver sections.

		VTG plasma concentration	
Estuary	**n=**	**Mean**	**Standard Deviation**
Alde	27	0.730	1.592
Humber	29	0.280	0.420
Medway	38	0.200	0.002
Thames	22	0.200	0.001
Tyne	28	101.800	398.838
Mersey	29	23.700	124.860

Table 2: Mean VTG plasma concentrations (µg ml^{-1}) for male *P. flesus* from all estuaries sampled during this study.

Location	Sampling Matrix	∑PBDE (µg kg^{-1})	∑HBCD (µg kg^{-1})	∑PCB (µg kg^{-1})	∑PAH (µg kg^{-1})	THC (µg kg^{-1})
Alde	Male	95.88	4.69	383.91	-	-
	Female	49.09	2.41	322.48	-	-
	Sediment	313.84	29.41	-	1866.85	174.00
Humber	Male	371.29	31.17	861.33	-	-
	Female	309.56	13.16	746.23	-	-
	Sediment	4509.46	198.76	-	5499.31	803.00
Medway	Male	286.47	124.82	2222.34	-	-
	Female	216.94	66.60	2489.17	-	-
	Sediment	1849.93	110.00	-	34850.88	1614.67
Thames	Male	679.63	298.83	2861.32	-	-
	Female	528.81	291.54	2642.94	-	-
	Sediment	8751.17	490.16	-	16209.30	1390.33
Tyne	Male	299.93	165.47	882.40	-	-
	Female	287.63	197.09	777.57	-	-
	Sediment	5060.43	624.77	-	25625.97	2857.67
Mersey	Male	117.82	61.62	2943.10	-	-
	Female	164.95	92.38	4647.99	-	-
	Sediment	12110.00	535.56	-	316.60	43.10

Table 3: Concentration of total contamination in pooled biota (male and female) and sediment (d.w.) samples. Data were normalised for lipid weight (l.w.) and total organic carbon (TOC) for biota and sediment, respectively (data for ∑PAH was not normalised). Total hydrocarbon content (THC) and PAH concentrations in estuarine sediment are presented as a mean of three replicates from each estuary. Data for individual congeners are available in supplementary tables S5-8.

Figure 1a

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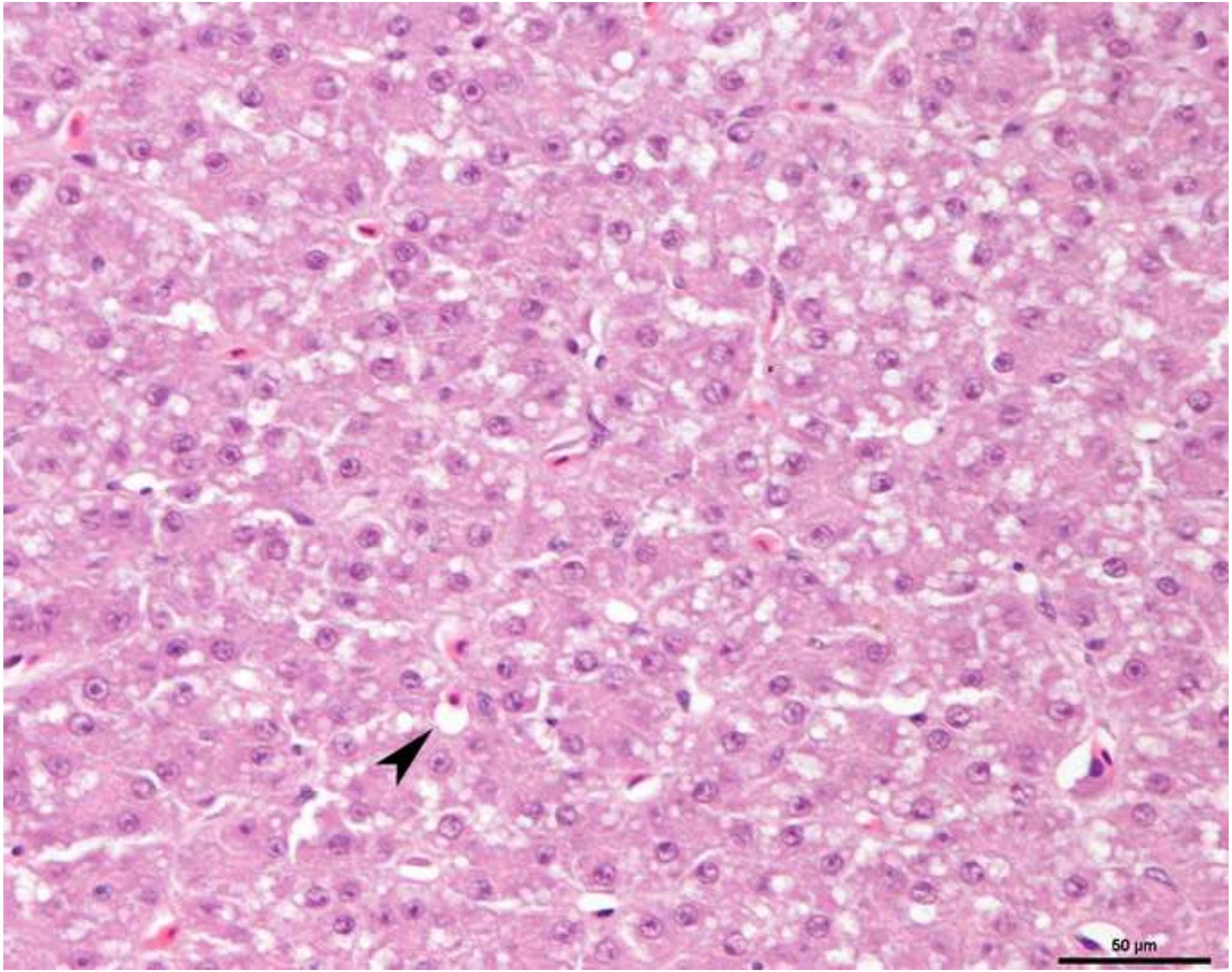

Figure 1b

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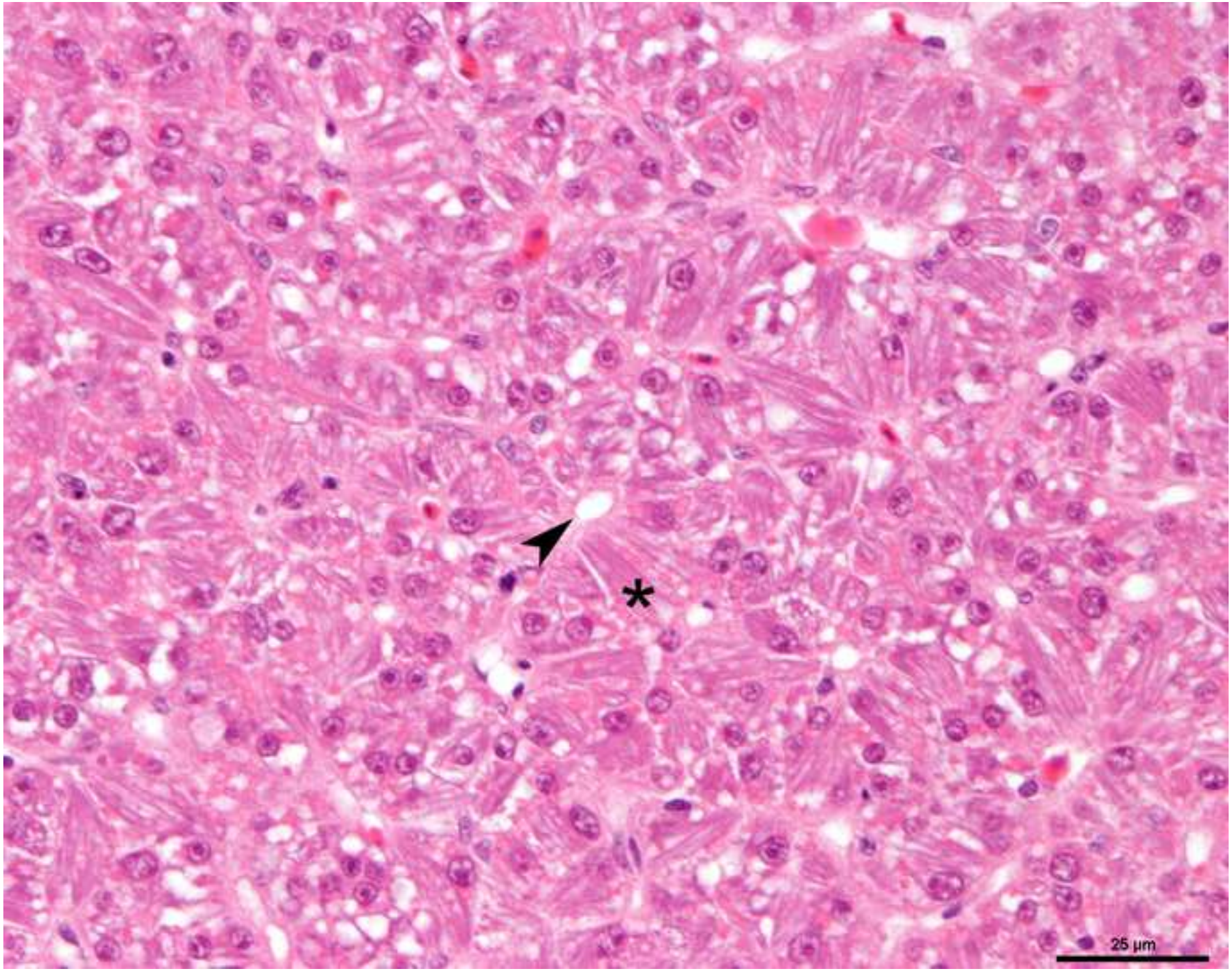

Figure 1c

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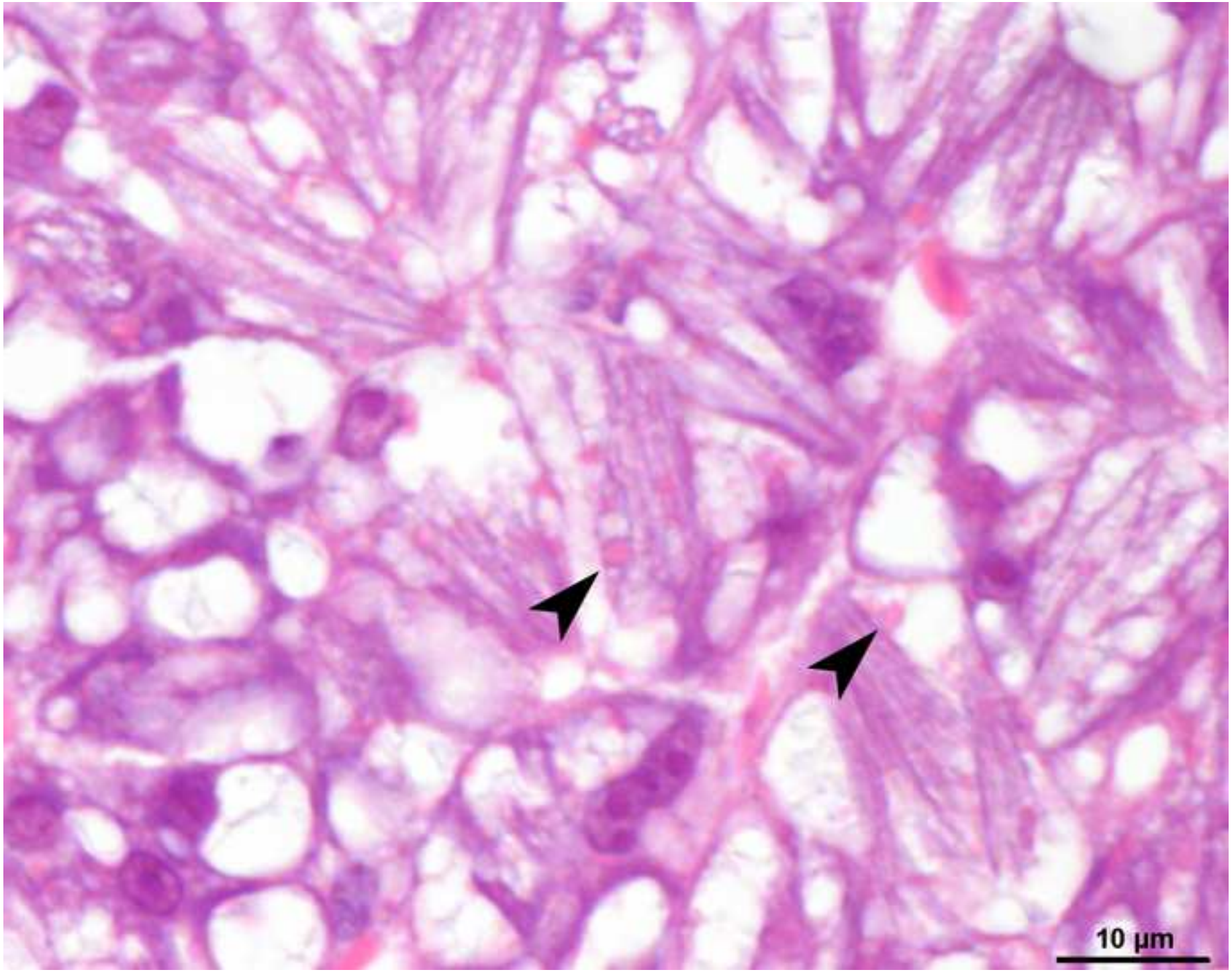

Figure 1d

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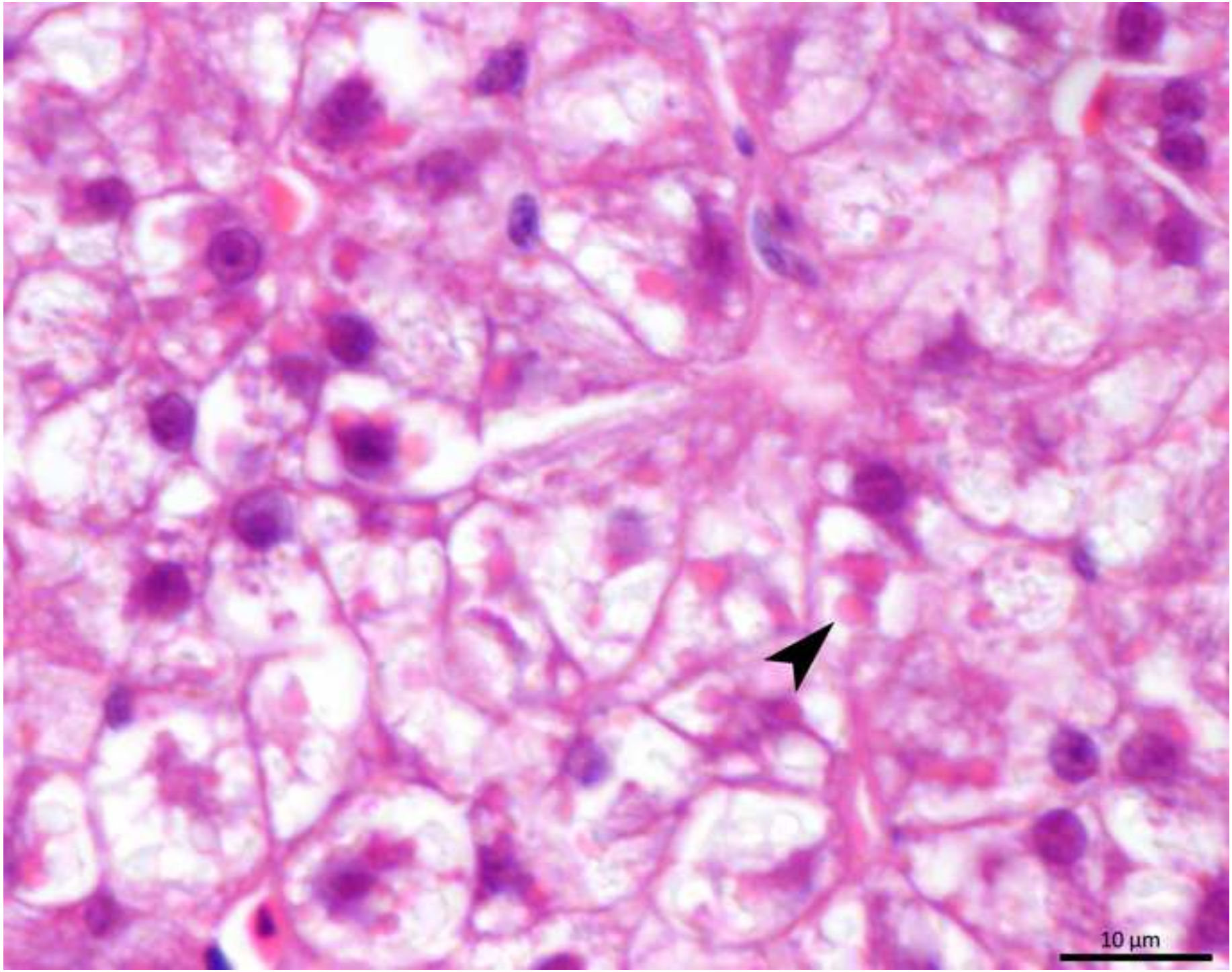

Figure 2a-b

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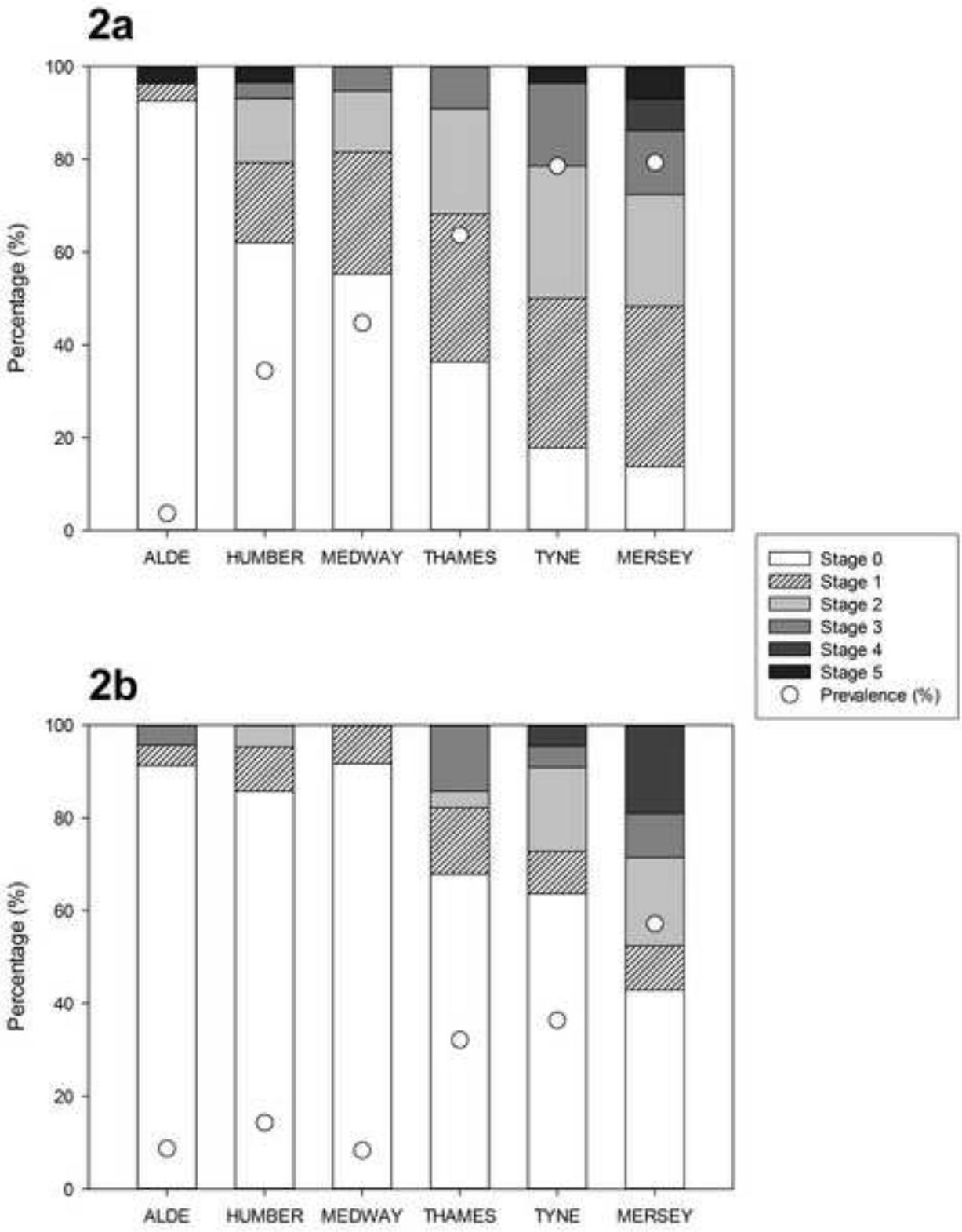

Figure 3a-b

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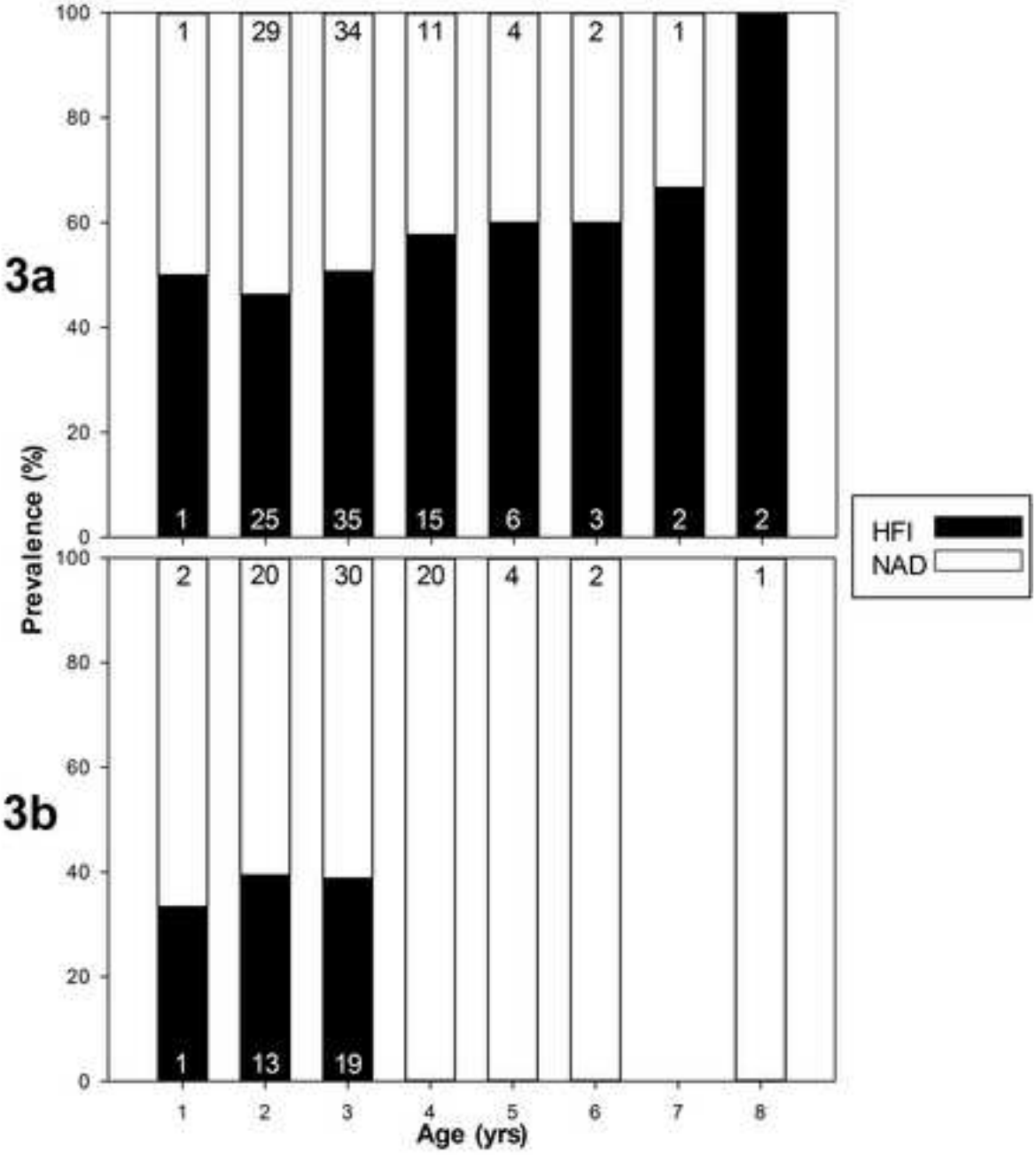

Figure 4a

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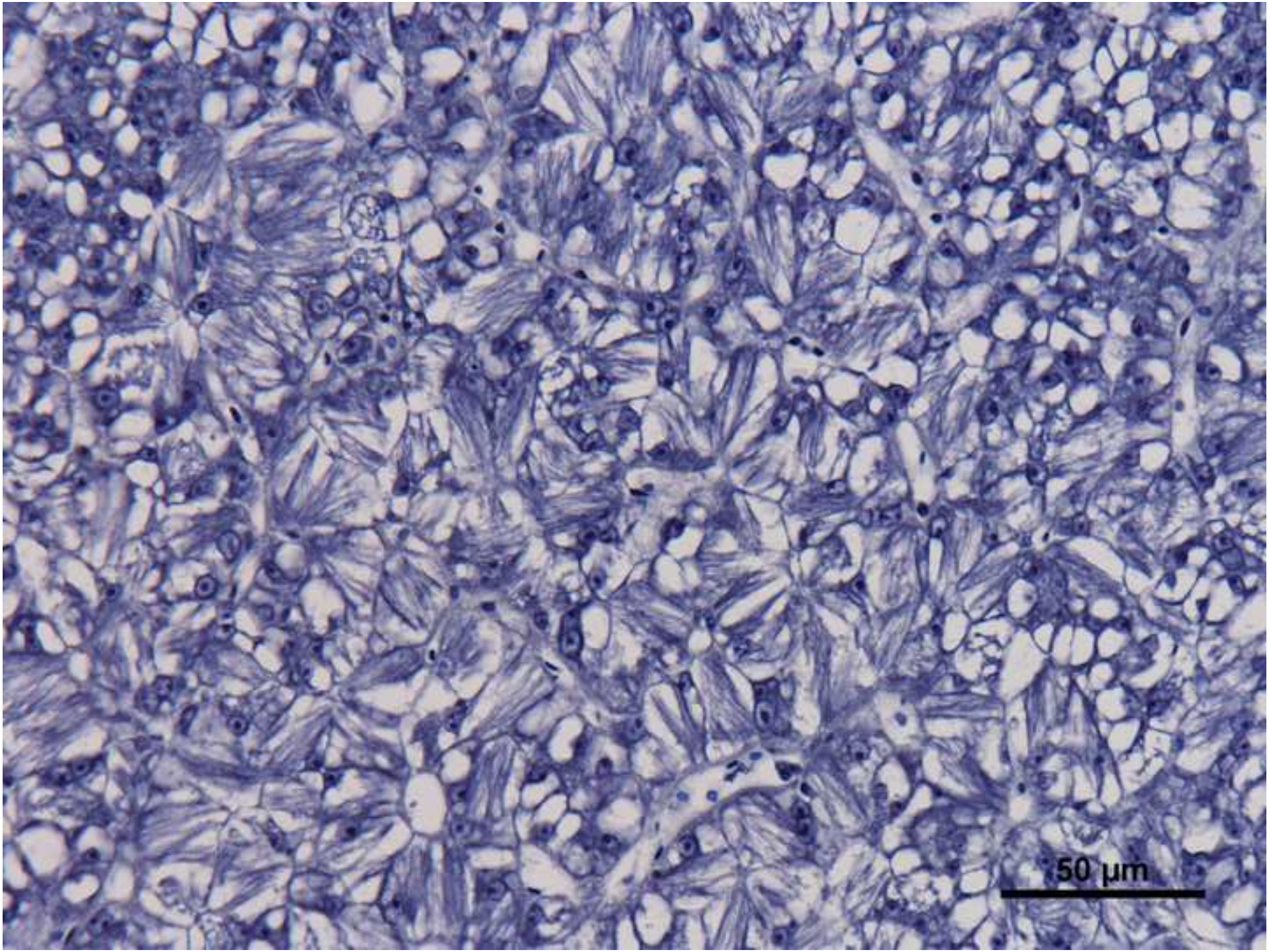

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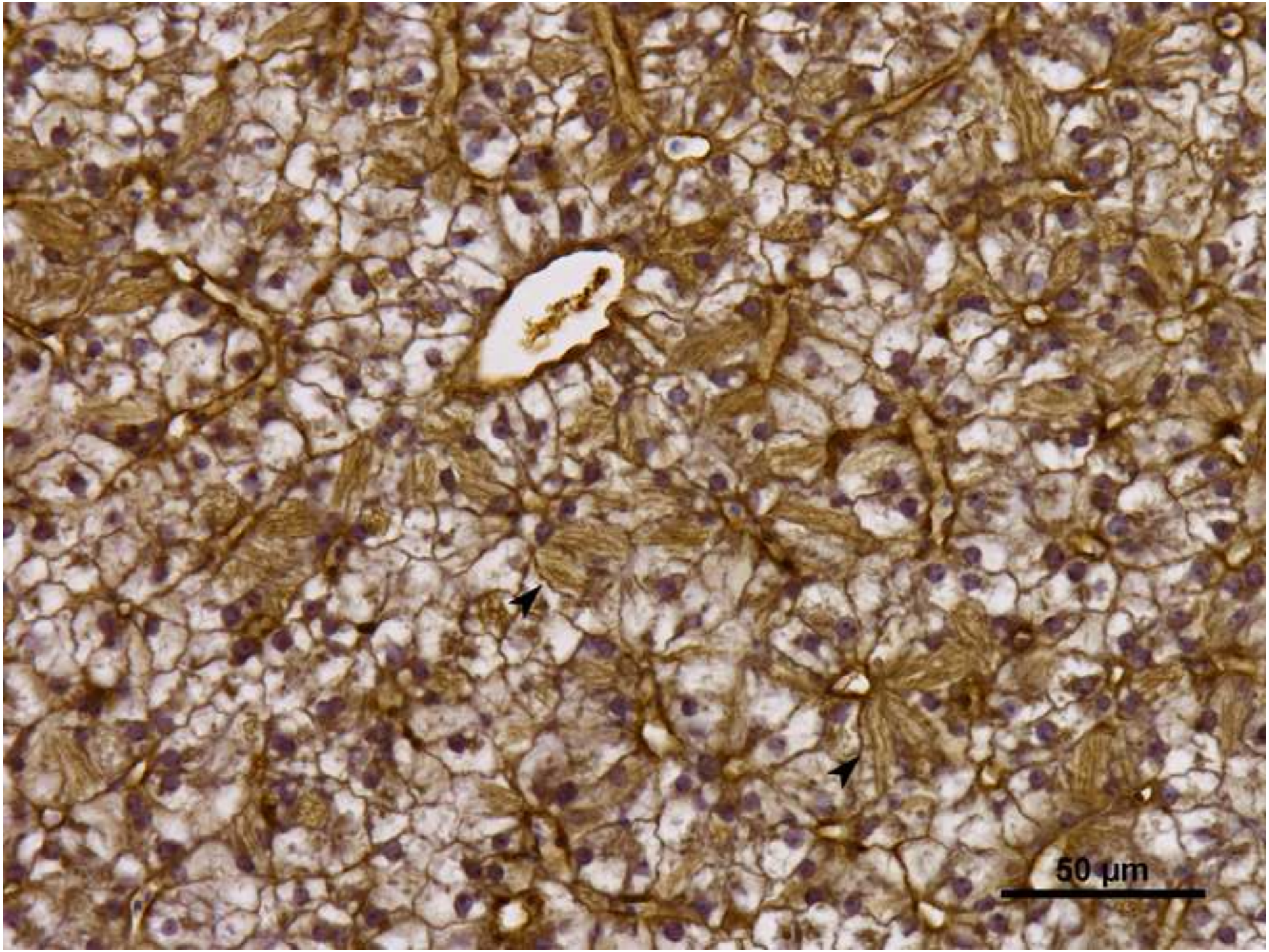

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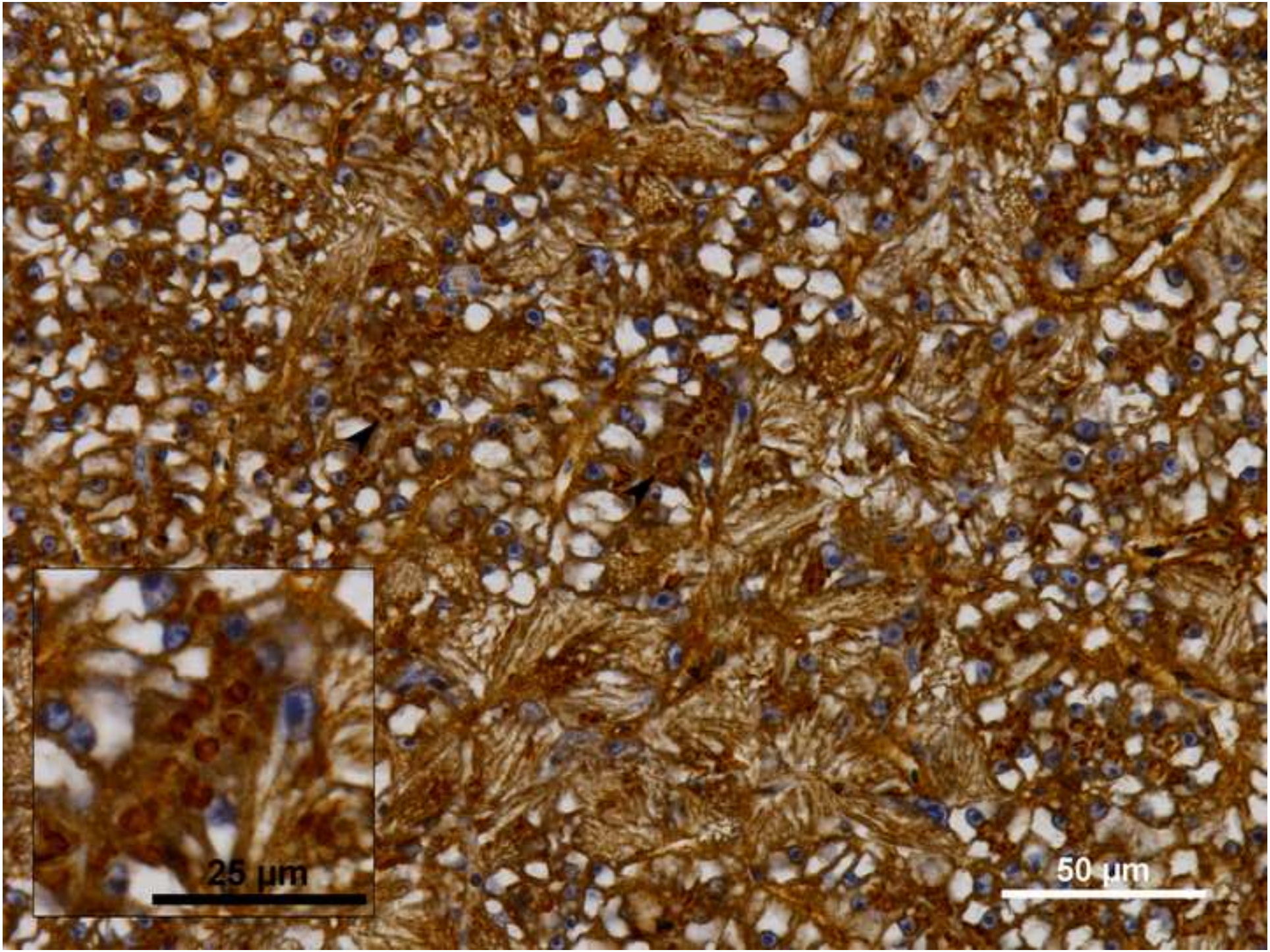

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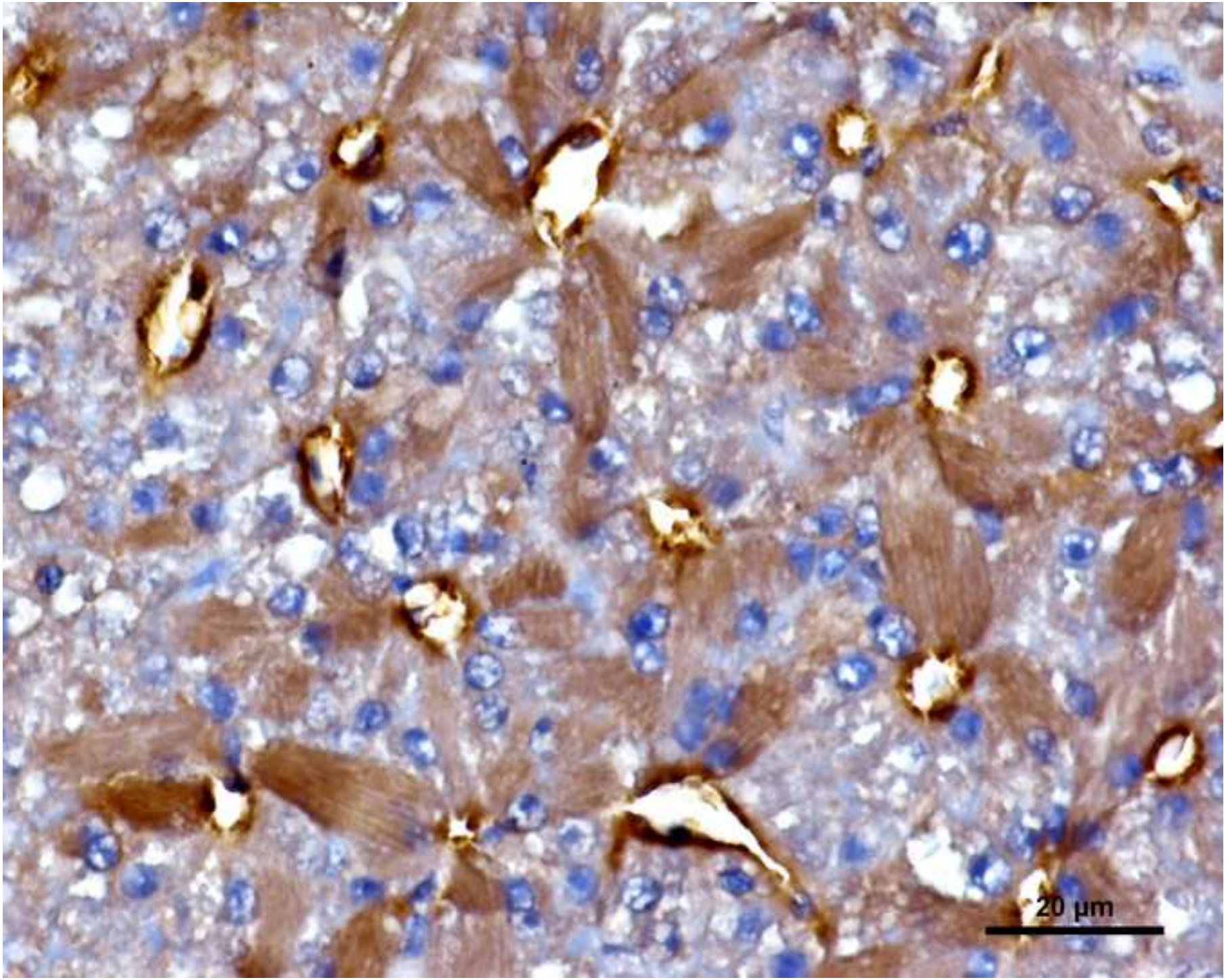

Figure 5a

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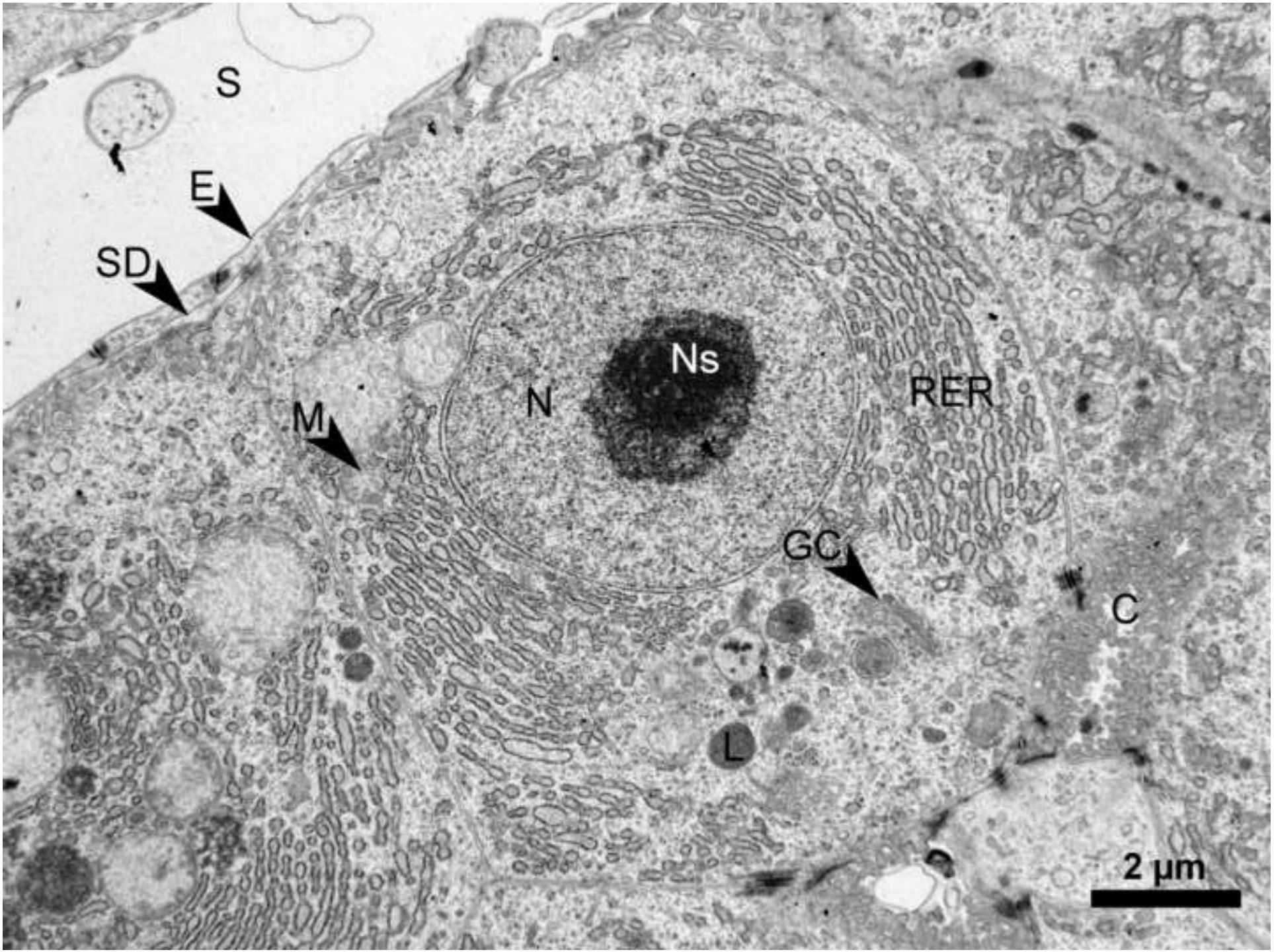

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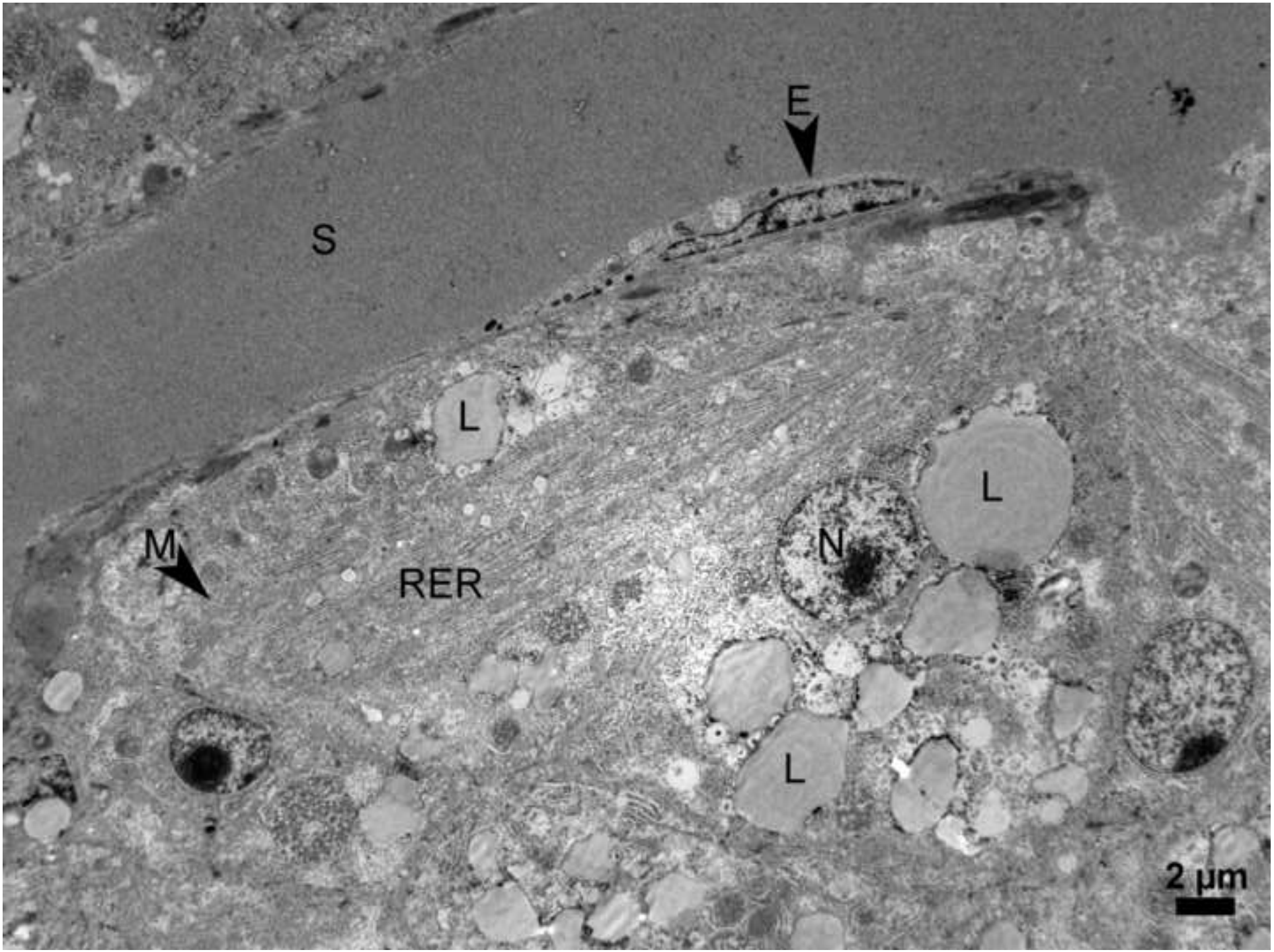

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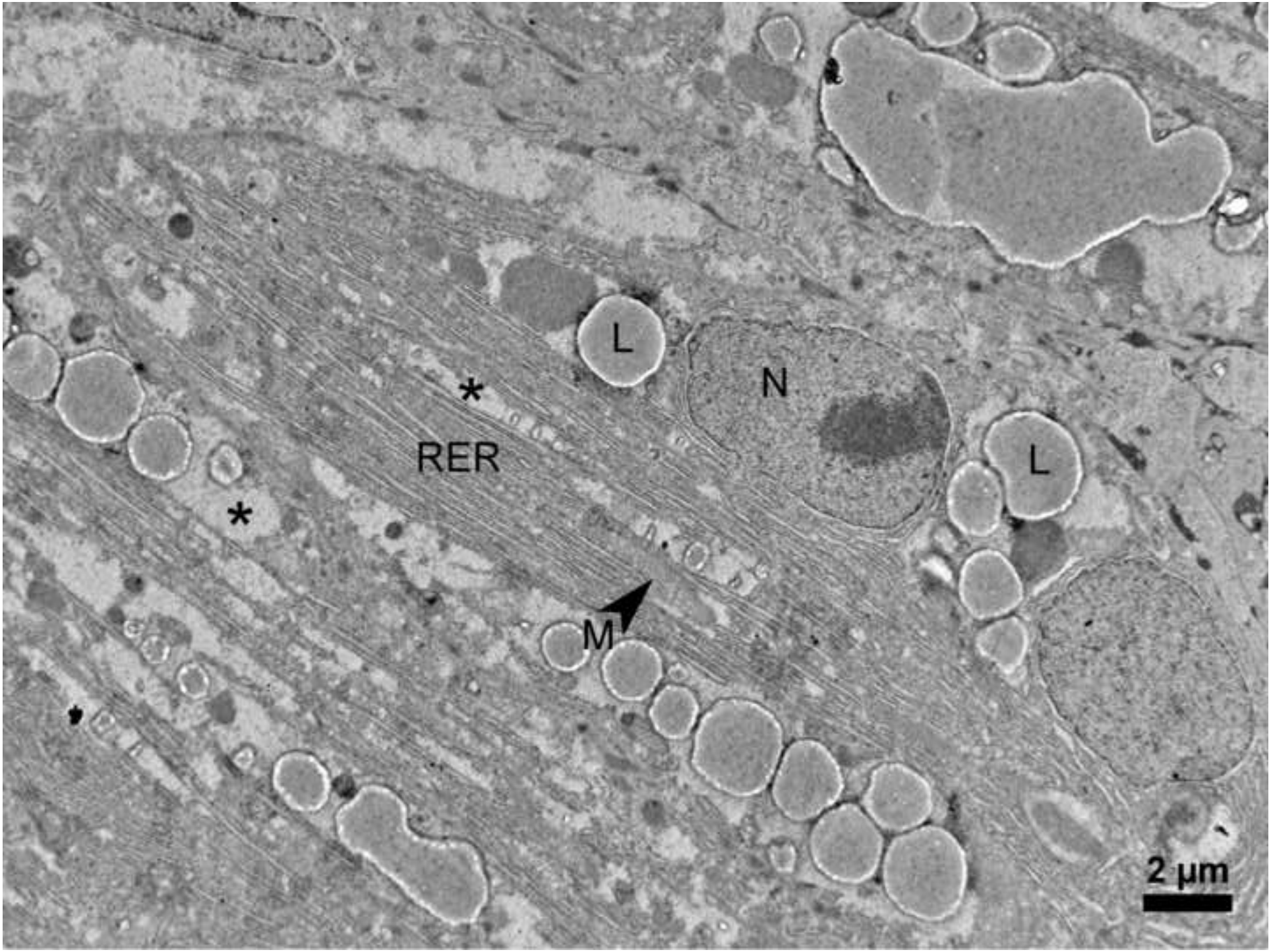

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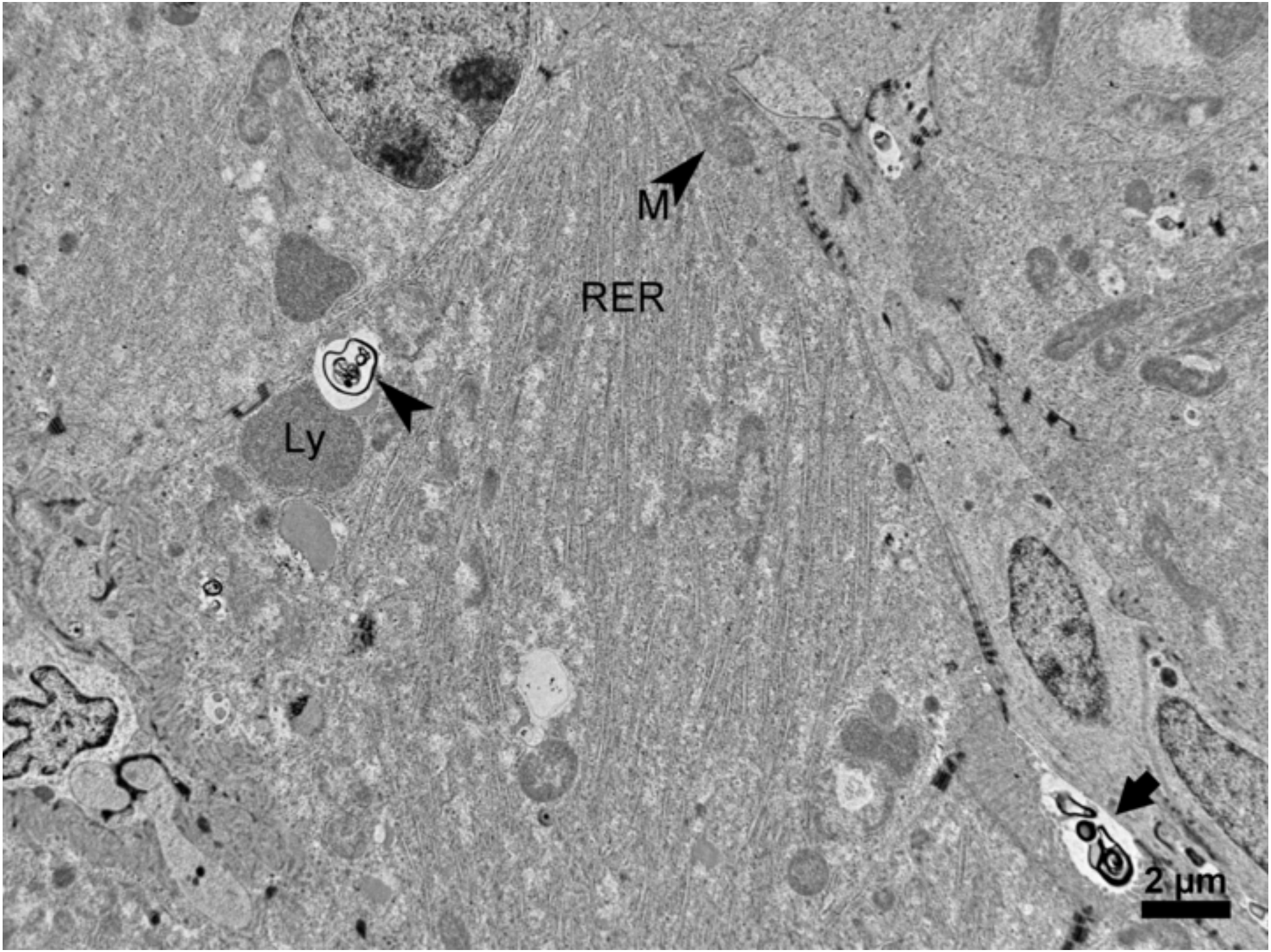

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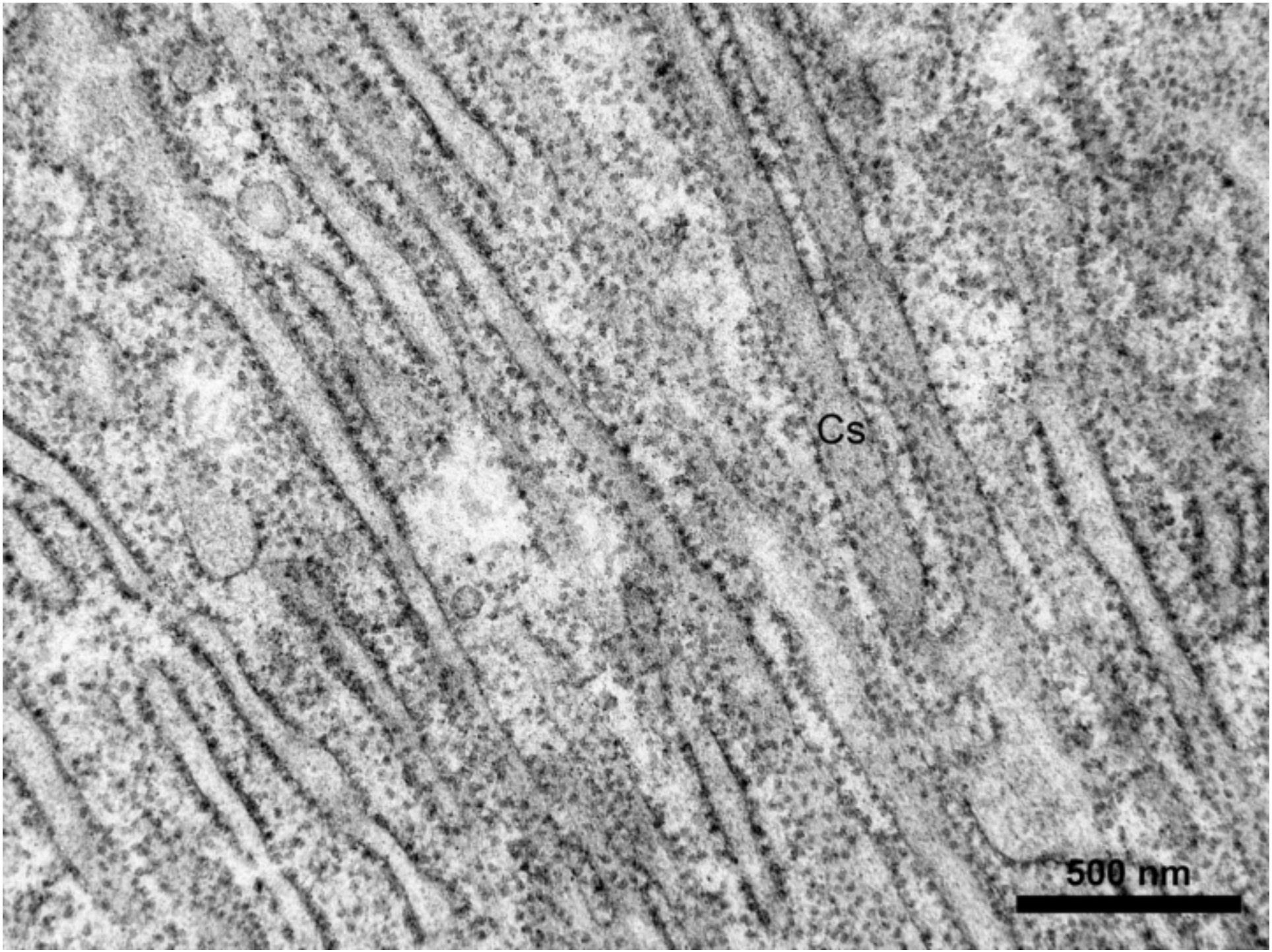

Figure 5f

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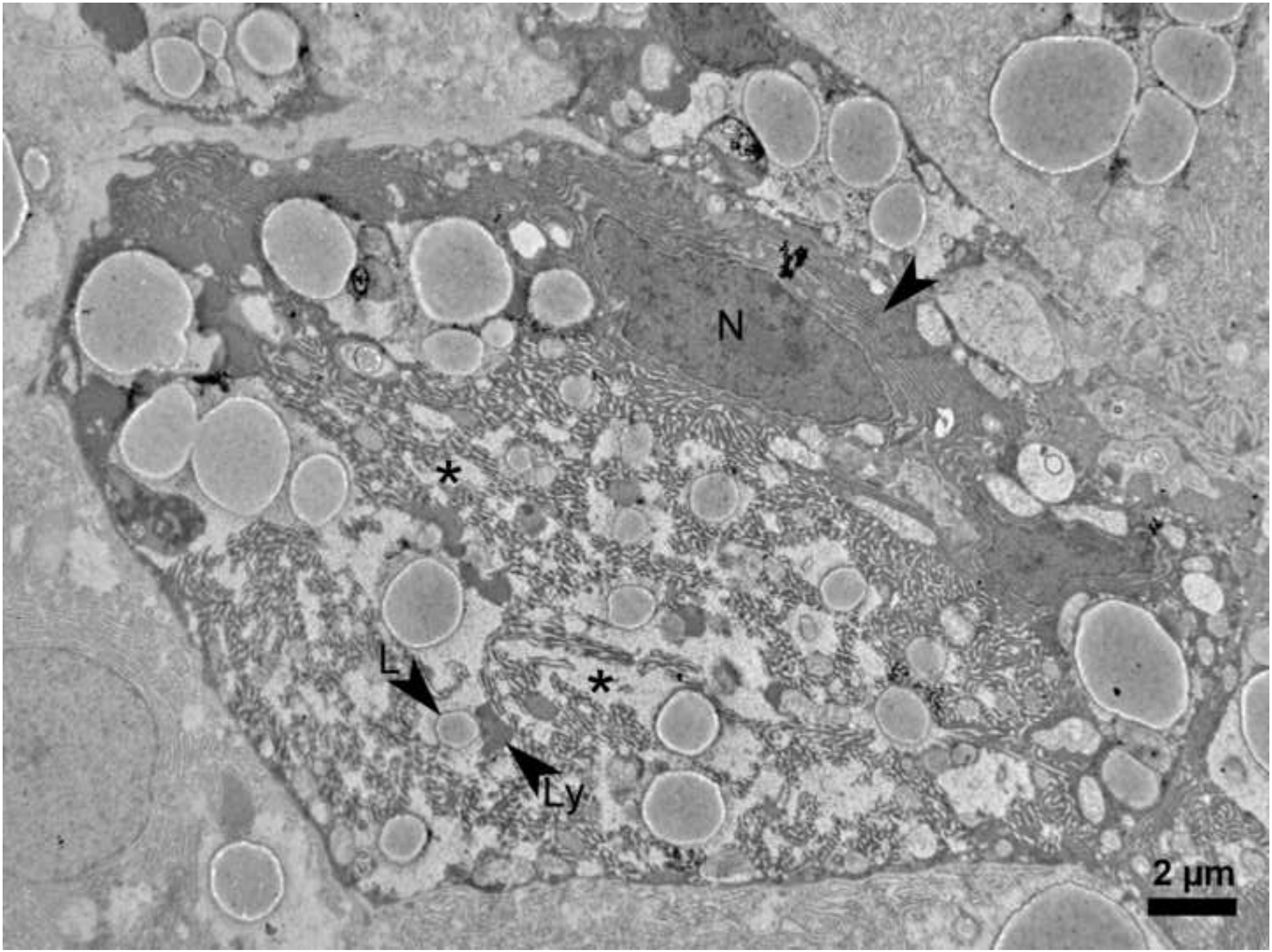

Figure captions

FIGURE AND SUPPLEMENTARY FIGURE LEGEND

Figure 1a: Normal histological section of male *P. flesus* liver from the River Alde with no abnormalities detected (NAD). Each sinusoid (arrowhead) was surrounded by a single layer of normal hepatocytes. HE. Scale bar, 50 µm. **Figure 1b:** HFI in female *P. flesus* from the River Mersey. HFIs here are characterised by significant numbers of pronounced longitudinal basophilic brush-like structures within hepatocytes (*). Sinusoid (arrowhead). HE. Scale bar, 25 µm. **Figure 1c:** Female *P. flesus* demonstrating presence of eosinophilic substance (arrowhead) located between fibrillar arrays. HE. Scale bar, 10 µm. **Figure 1d:** Male *P. flesus* from River Mersey demonstrating pronounced eosinophilic substance (arrowhead) located within hepatocytes. The quantity of eosinophilic substance appeared more prevalent in cells containing fewer HFIs. HE. Scale bar, 10 µm.

Figure 2a: 100% stacked column chart demonstrating prevalence and proportion of male *P. flesus* exhibiting each HFI severity stage described in table 1. HFI stage 5 not included in calculation of prevalence. **Figure 2b:** 100% stacked column chart demonstrating prevalence and proportion of female *P. flesus* exhibiting each HFI severity stage described in table 1. HFI stage 5 not included in calculation of prevalence. *The following figures in parenthesis show percentage sex ratio data for male and female respectively: Alde (54, 46), Humber (58, 42), Medway (76, 24), Thames (44, 56), Tyne (56, 44), Mersey (58, 42).

Figure 3: Age distribution stacked column chart showing proportion of **(a)** male and **(b)** female *P. flesus* of all ages from all sampling locations exhibiting HFI and no abnormalities detected (NAD). The quantity of affected and non-affected fish pertaining to each age class is shown directly on each data series.

Figure 4a: Negative control (no primary antibody) for IHC labelling of VTG. Scale bar, 50 µm. **Figure 4b:** IHC labelling of VTG in 5-year-old male *P. flesus* using polyclonal anti-rabbit *P. flesus* VTG antibody (1:5000). Positively labelled VTG demonstrated immediate association with HFIs. Individual HFI fibrils can clearly be seen within hepatocytes (arrowhead). Scale bar, 50 µm. **Figure 4c:** IHC labelling of VTG in 3-year-old female *P. flesus* using polyclonal anti-rabbit *P. flesus* VTG antibody (1:5000). Positively labelled VTG demonstrated immediate association with HFIs. IHC appeared to reveal positive labelling of previously identified eosinophilic substance present in hepatocytes (arrowhead). Scale bar, 50 µm (Inset scale bar, 25 µm)**. Figure 4d:** IHC labelling of VTG in 2-year-old male *P. flesus* from Mersey using polyclonal anti-rabbit *P. flesus* VTG antibody (1:5000). Positive labelling was specific with little background, as demonstrated by restriction to those hepatocytes affected with HFI (*). Scale bar, 20 µm.

Figure 5a: Transmission electron microscopy (TEM) of normal *P. flesus* hepatocyte. The nucleus (N) is surrounded by stacks of rough endoplasmic reticulum (RER). Nucleolus (Ns), the Golgi complex (GC), sinusoid (S), mitochondrian (M), space of Dissé (SD), lysosome (Ly), sinusoid endothelium (E), caniculus (C). Scale bar, 2 µm. **Figure 5b:** Transmission electron microscopy (TEM) of HFI affected *P. flesus* hepatocyte. HFI are confirmed here as rough endoplasmic reticulum (RER) orientated across full axis. Much of the hepatocellular content is displaced towards the periphery of cell. Nucleus (N), sinusoid (S), mitochondrian (M), sinusoid endothelium (E), lipid-like substance (L). Scale bar, 2 µm**. Figure 5c:** Rough endoplasmic reticulum (RER) orientated across full axis interspersed with mitochondria (M), lysosomes (Ly), and lipid-like substance (L). Note the immediate association of lysosomes (Ly) with lipid-like substance. Vacuous spaces between apparent disintegrating RER were frequently observed (*). Scale bar, 2 µm. **Figure 5d:** Hepatocyte affected with HFI. Note coalescence of lysosome (Ly) with autophagasome (accompanying arrowhead) containing membranous material. Occasionally, similar material appeared to have been ejected into the bile canaliculi (arrow). Rough endoplasmic reticulum (RER), mitochondrian (M). Scale bar, 2 µm. **Figure 5e:** Detail of HFI revealed ribosomes (arrow) studded along full length of RER cisternae (Cs). Scale bar, 500 nm. **Figure 5f:** Hepatocytes occasionally demonstrated regions of both intact (arrowhead) and disintegrating RER (*) with "moth-eaten" appearance. Note the immediate association of lysosomes (Ly) with lipid-like substance (L). Scale bar, 2 µm.

Supplementary Figure 1: Box and whisker plot demonstrating relative quantity of VTG and CHR gene transcripts in male *P. flesus* from the Tyne and Mersey for each biological group (HFI severity stage). No significant differences were observed between biological groups.

Supplementary Figure 2: Relationship of VTG plasma concentrations (limit of detection= 0.2 µg ml^{-1}) and VTG transcript levels (relative to female control=1) of male *P. flesus* sampled from the Tyne and Mersey estuaries. The data show two distinct regions comprised of 'baseline' and 'elevated'. The following data in parenthesis indicate VTG plasma concentration, VTG transcript levels and HFI severity stage respectively, between four *P. flesus* exhibiting relatively high VTG concentrations: A (897.9, 0.038, 0), B (1944.0, 0.200, 5), C (672.9, 0.600, 2), D (8.7, 0.124, 2).