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LABORATORY OF VETERINARY MORPHOLOGY, GHENT UNIVERSITY

# MACROPHAGE ACCUMULATION IN DEEPER REGIONS OF EQUINE EXUBERANT GRANULATION TISSUE SUGGESTS PERSISTENT FIBROPROLIFERATIVE SIGNALING

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## BACKGROUND

- Exuberant granulation tissue (EGT) = second intention **wound healing disorder in distal limbs of horses** with fibroproliferative character[A, B]
- Unknown pathomechanism and lack of evidence-based treatment options[B, C]
- Vicious cycle of **recurrent inflammation and proliferation** within wound healing cascade[D]
- Dysregulated macrophage infiltration & polarisation suggested to drive fibroproliferation[E, F]
- Two different zones in the wound bed:

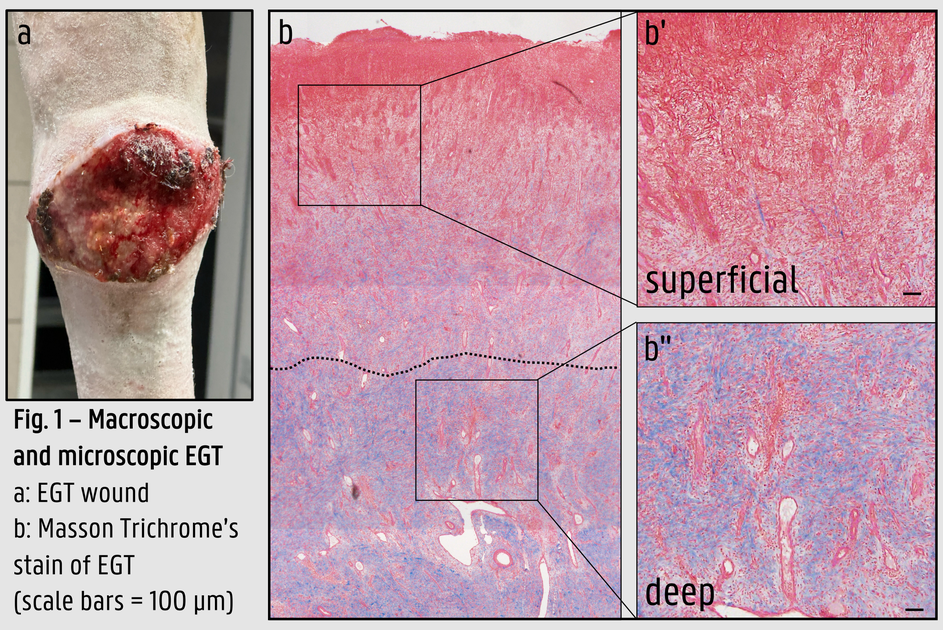



**Fig. 1 – Macroscopic and microscopic EGT**
a: EGT wound
b: Masson Trichrome's stain of EGT
(scale bars = 100 µm)

### Inflammatory layer

- Massive immune cell infiltration[D]
- Immature Collagen type III[G]

### Fibrotic layer

- High rate of neoangeogenesis with microvascular occlusion and endothelial cell hypertrophy[H]
- Mature Collagen type I[G]

## AIM OF THE STUDY

- Investigation of **spatial macrophage distribution** using pan-macrophagic marker Iba1 (immunohistochemistry)
- **Phenotypic characterisation of macrophages** using double staining of Iba1 and Vimentin as cytoskeletal marker (immunofluorescence)

## MATERIAL & METHODS

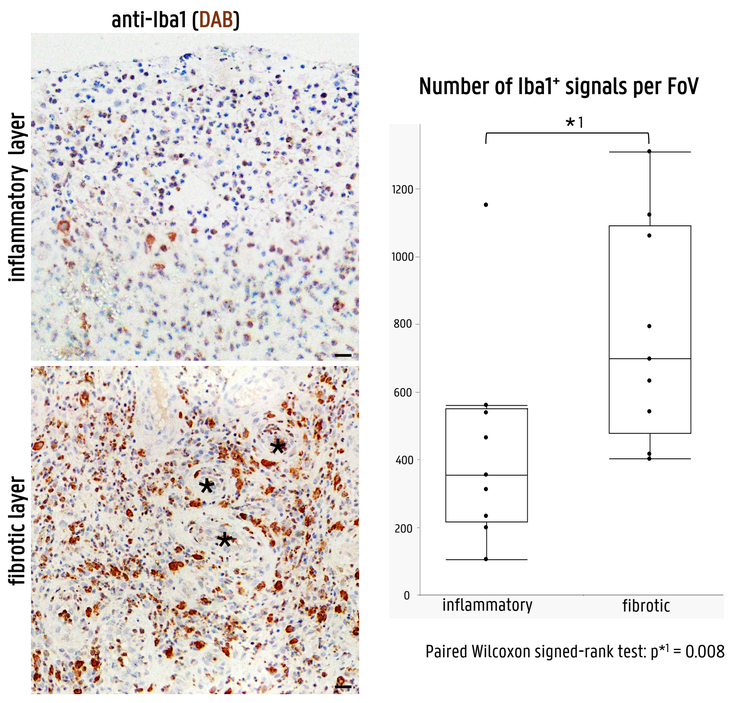
- FFPE sections of surgically trimmed EGT ($n_{EGT}$ = 11) & biopsies of experimentally induced distal limb wounds ($n_{control}$ = 5)

**Tab. 1** Overview staining protocols

| | Immunohistochemistry (IHC) | Immunofluorescence (IF) |
|---|---|---|
| **Number of EGT horses** | 9 | 11 |
| | **Staining procedure** | |
| **Pretreatment** | Dewaxing and rehydration with xylene and decreasing alcohol series | |
| **Antigen retrieval** | Citrate buffer (pH 6.0) in decloaking chamber 30 min at 90°C | |
| **Permeabilisation and blocking** | 30% goat serum for 30 min | 0.1% Triton TX 100 with 10% goat serum for 30 min |
| **Endogenous peroxidase block** | 3% hydrogen peroxide in methanol | - |
| **Primary antibodies** | Anti-Iba1 (rabbit polyclonal) 1:100 o.n. at. 4°C (Fujifilm cat. no. 019-19741) | Anti-Iba1 (rabbit polyclonal) 1:100 o.n. at 4°C (Fujifilm, cat. no.019-19741) Anti-Vimentin (conjugated to Cy3, mouse monoclonal) 1:500 for 2 h at RT (Sigma Aldrich, cat. no. C9080) |
| **Secondary antibodies & visualisation** | EnVision+™ Single Reagents, HRP. Rabbit for 30 min (cat. no. K4003) EnVision™ FLEX DAB+ Substrate Chromogen System for 5 min (cat. no. GV825) | AlexaFluor® Goat Anti-Rabbit 1:500 for 90min at RT (Invitrogen, cat. no. A11008) |
| **Nuclear counterstain** | Haematoxylin for 30 s | Hoechst® 33342 1:1000 in PBS for 10 min |
| **Mounting** | Rehydration with increasing alcohol series and xylene DPX | Fluoroshield™ |
| | **Image acquisition** | |
| **Fields of view (Inflammatory and fibrotic)** | 3 per layer 0.25 mm^2 | 5 per layer 0.607 mm^2 |
| | **Image analysis** | |
| **Software** | QuPath | Fiji ImageJ |
| **Statistics** | JMP® Inflammatory vs. fibrotic EGT - paired Wilcoxon signed-rank test Overall comparison with control: Kruskal-Wallis & Mann-Whitney U test with Bonferroni correction | |

## RESULTS

### Spatial distribution

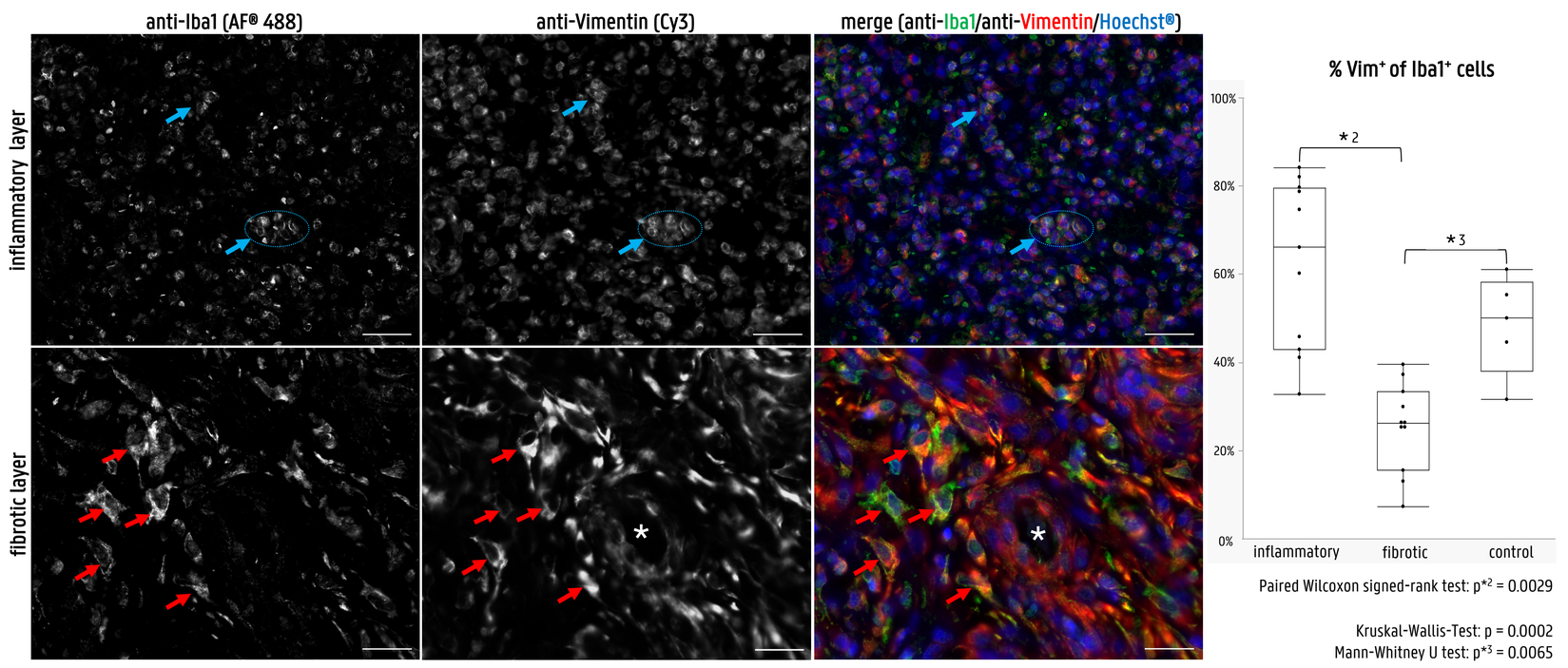



Paired Wilcoxon signed-rank test: $p^{*1} = 0.008$

**Fig. 2 – QuPath analysis of IHC reveals increased Iba1⁺ signal density in fibrotic EGT**

- Fibrotic macrophages display more polymorphic morphology with vessel association (*)

### Phenotypic characterisation




Paired Wilcoxon signed-rank test: $p^{*2} = 0.0029$

Kruskal-Wallis-Test: p = 0.0002
Mann-Whitney U test: $p^{*3} = 0.0065$

**Fig. 3 – Fiji ImageJ analysis of IF reveals increased Vim⁺/Iba1⁺ cell number in inflammatory regions**

- Significantly higher percentage of Vim⁺/Iba1⁺ cells in inflammatory versus fibrotic region & control group versus fibrotic region
- Vessel association (*) and polymorph morphology of fibrotic region macrophages (→) consistent with IHC
- Fibrotic region macrophages (→) appear to have larger cell volume compared to inflammatory macrophages (→)

scale bars = 25 µm

## DISCUSSION & OUTLOOK

➢ **Spatial macrophage heterogeneity**: Morphology and Vimentin expression of macrophages differ within EGT lesions suggesting altered polarisation

➢ **Inflammation as driver of cytoskeletal activation:** Increased number of Iba1⁺/Vim⁺ cells in inflammatory regions

Future perspective: *In vitro* co-culture of PBMC-derived M1/M2-like macrophages with EGT myofibroblasts to assess phenotype-specific effects on cell behaviour

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