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# A Disintegrin and Metalloproteinase with Thrombospondin Motifs-4 (ADAMTS-4) levels in chondrocytes of different morphology within non-degenerate and early osteoarthritic human femoral head cartilage

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# Cartilage

**A Disintegrin and Metalloproteinase with Thrombospondin Motifs-4 (ADAMTS-4) levels in chondrocytes of different morphology within non-degenerate and early osteoarthritic human femoral head cartilage.**

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|------------------|---|
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Manuscripts

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2 **A Disintegrin and Metalloproteinase with Thrombospondin Motifs-4 (ADAMTS-4) levels in**  
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4 **chondrocytes of different morphology within non-degenerate**  
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6 **and early osteoarthritic human femoral head cartilage.**  
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10 **Introduction and Summary.**  
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12 Osteoarthritis (OA) is a common degenerative disorder affecting whole synovial joints.  
13 Characteristic features include progressive loss of mechanically-weakened cartilage which  
14 eventually exposes sub-chondral bone, and movement results in extreme pain and ultimately joint  
15 failure<sup>1</sup>. Understanding early extracellular matrix (ECM) damage is a key research objective with  
16 aggrecan degradation a hallmark of OA<sup>2</sup>. Early OA shows strong immunostaining for the major pro-  
17 inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  suggesting an important role in OA<sup>3</sup>. These stimulate  
18 production of two groups of proteolytic enzymes, the MMPs (metalloproteinases) and the ADAMTS  
19 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) family both which cause  
20 aggrecan degradation. ADAMTS-4 (aggrecanase-1<sup>4</sup>) and ADAMTS-5 have been implicated since  
21 aggrecan fragments in OA match those produced by these enzymes<sup>5</sup>. ADAMTS-4 is induced by IL-  
22 1 $\beta$  and TNF- $\alpha$  whereas ADAMTS-5 is constitutively expressed in normal cartilage<sup>6</sup>. Relative mRNA  
23 levels of ADAMTS-4 are ~4-fold higher in knee OA compared to normal cartilage and correlate with  
24 degenerative progression<sup>7</sup>. However, inhibitory elements may be upregulated and post-  
25 transcriptional modifications occur, so this does not necessarily constitute higher ADAMTS-4 protein  
26 levels. Although ADAMTS-4 knockouts showed no effect against cartilage degeneration in an animal  
27 instability model<sup>8</sup>, the pathway(s) for cartilage loss in idiopathic human OA may be different.  
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46 Early microscopic changes to chondrocytes in otherwise normal cartilage, might herald the  
47 start of the vicious cycle of OA. Imaging fluorescently-labelled *in situ* human chondrocytes by  
48 confocal scanning laser microscopy (CLSM), identified a small, but potentially important population  
49 of abnormally-shaped chondrocytes<sup>9,10</sup>. They exhibit cytoplasmic processes, the cells resembling a  
50 fibroblastic rather than a chondrocytic phenotype<sup>11</sup> which might reflect or lead to deleterious matrix  
51 metabolism<sup>12</sup>. Abnormal chondrocyte morphology correlated with increased cell-associated IL-1 $\beta$ ,  
52 decreased chondron-localised collagen type VI<sup>13</sup> and increased collagen type I<sup>14</sup>. It is possible that  
53 IL-1 $\beta$  stimulation results in localised aggrecan depletion, the development/acceleration of abnormal  
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1 morphology and a fibro-cartilagenous phenotype. Thus in non-degenerate human cartilage, these  
2 peculiar chondrocytes may possess increased ADAMTS-4 levels in contrast to normal  
3 (elliptical/rounded) chondrocytes. Alterations to ADAMTS-4 levels associated with chondrocyte  
4 clusters is also of interest as proliferation is a characteristic of later OA and could cause additional  
5 ECM weakness.

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12 Using CLSM imaging, fluorescently-labelled *in situ* chondrocytes were classified cell-by-cell  
13 into those with (a) normal morphology, (b) abnormal morphology ( $\geq 1$  process/cell) and (c) cells ( $\geq 4$ )  
14 in a cluster, within grade 0 (G0; normal non-degenerate) and grade 1 (G1; mild OA) human cartilage.  
15 On the same cells, we semi-quantitatively assessed ADAMTS-4 immuno-fluorescence (IF) and  
16 determined its relationship with chondrocyte morphology. The results demonstrated marked  
17 heterogeneity in the morphology and ADAMTS-4 levels of human chondrocytes. In macroscopically-  
18 normal cartilage, a small but significant population of abnormal chondrocytes was present  
19 possessing higher levels of ADAMTS-4 compared to normal or clustered cells. These early changes  
20 to ADAMTS-4 levels might indicate ECM regions more sensitive to mechanical load and focal points  
21 for OA cartilage degeneration.

## 32 33 34 35 36 **Methods, Results and Discussion**

37 Femoral heads (FHs) were obtained from N=14 patients (8 females; 6 males (range 44-89yrs; mean  
38 71yrs)) undergoing surgery for femoral neck fracture. Ethical permission (Tissue Governance (NHS),  
39 Lothian) and consent were obtained. Cartilage areas were visually graded G0 or G1 using OARSI  
40 criteria and explants taken<sup>11</sup>. Chondrocyte morphology was identified using 5-  
41 chloromethylfluorescein-diacetate (CMFDA). Spheroidal/elliptical cells without cytoplasmic  
42 processes were classified as normal cells. A cytoplasmic process was defined as a CMFDA-labelled  
43 protrusion of  $\geq 2\mu\text{m}$ . Chondrocyte clusters possessed  $\geq 4$  cells within the lacunar space. Sections  
44 were incubated with ADAMTS-4 antibody (PA1-1749A, Invitrogen; 1:100), then incubated with  
45 secondary antibody (AlexaFluor™ ab175471, Abcam; 1:200). Sections were mounted (propyl-  
46 gallate; Sigma-Aldrich, Dorset), imaged (Zeiss LSM800) and analysed (Imaris™, Oxford Instruments,  
47 U.K.). A section from each explant was used as a negative control with background secondary  
48 antibody fluorescence subtracted to identify specific labelling. A cell-by-cell mask selection allowed  
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2 analysis of cell-specific (punctate) immuno-fluorescence which localised with CMFDA labelling. For  
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4 cell clusters, the selection encompassed the whole cluster and fluorescence assessed on a 'per cell'  
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6 basis. Fluorescence intensity was presented in Relative Fluorescence Units (RFU). Asterisks  
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8 represent  $P \leq 0.05^*$ ,  $P \leq 0.01^{**}$ , and  $P \leq 0.001^{***}$ . Femoral heads (N) and cells (n) were presented as  
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10 [N(n)].  
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12 There were clear differences between cells in G0 and G1 cartilage (Fig.1A(a,b)). At low  
13  
14 magnification, the majority of cells in G0 were normal, however at high magnification, abnormal  
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16 chondrocytes were observed (Fig.1A(a)(iii)). In G1, clustering and cytoplasmic processes were  
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18 detected at low and high magnification (Fig.1A(b)(i-iv)). Chondrocyte morphology classified into  
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20 normal, abnormal or clusters was performed by eye. There was a decrease (90% to 50%) in normal  
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22 chondrocytes between G0 and G1 ( $P < 0.01$ ; Fig.1B(a)). However, there was ~2-fold increase in  
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24 abnormal cells ( $P < 0.05$ ; Fig.1B(b)), and a >6-fold increase in cells within a cluster  
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26 ( $P < 0.001$ ; Fig.1B(c)). Occasionally, these cells exhibited processes (Fig.1A(b)(ii)) and were classified  
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28 as a cell in a cluster. The average length of processes increased from  $3.81 \pm 0.67 \mu\text{m}$  to  $6.44 \pm 1.16 \mu\text{m}$   
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30 (G0 vs G1 cartilage [4(77);  $P = 0.0125$ ]).  
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33 Having identified the chondrocyte morphologies, their association with ADAMTS-4 levels was  
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35 assessed. There was clear ADAMTS-4 immuno-fluorescence (Fig.2A(ii&iv)) which matched the  
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37 CMFDA-labelled cells, appearing as punctate labelling closely delineating the same size/morphology  
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39 suggesting cell-associated labelling (Fig.2A(i&iii)). CLSM visualisation highlighted the  
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41 heterogeneous nature of ADAMTS-4 labelling. Both diffuse and punctate labelling was evident with  
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43 strong labelling in some cells close to weakly-labelled cells (Fig. 2A(ii&iv)). For all cell types assessed  
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45 in either G0 or G1 cartilage, there was no difference between mean or punctate fluorescence levels  
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47 ( $P = 0.5245$ ;  $P = 0.4080$  respectively). This indicated mean levels over a relatively large area or when  
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49 measured as cell-associated protein did not change with early cartilage degeneration. However cell-  
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51 associated (punctate) labelling was higher ( $P < 0.001$ ) in both G0 and G1 compared to mean levels  
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53 and this was expected since it related to cell-specific labelling which covered smaller and more  
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55 intense areas of labelling. Clearly, this generalised chondrocyte labelling could mask the  
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57 heterogeneous distribution of ADAMTS-4 between the various morphologies (Fig.1A). Accordingly,  
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59 >4000 cells within G0 and G1 cartilage were imaged, and morphology and ADAMTS-4  
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1 immunofluorescence analysed cell-by-cell. Despite the data spread, abnormal cells demonstrated  
2 higher intensity than normal ( $P=0.0440$ ) or clustered chondrocytes ( $P<0.0001$ ;Fig.2B(i)).  
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6 With cartilage degeneration, there was a marked increase in the proportion of abnormal cells  
7 and clustered cells (Fig.1B(a-c)). Thus in G1 cartilage it was possible increased numbers of  
8 abnormal cells with greater levels of ADAMTS-4 would be offset by the increased number of cell  
9 clusters which had lower levels of the enzyme. This could account for the lack of a difference  
10 between overall levels of the enzyme between G0 and G1 noted above. Accordingly, the  
11 percentages of chondrocytes in G0 and G1 cartilage with a given IF intensity were plotted (Fig.2B(ii)).  
12 While there was a broad distribution in both grades, the levels in G0 cartilage (with normal and  
13 abnormal cells, but negligible clusters) was higher and broader than that in G1 cartilage. For G1  
14 cartilage, all three cell categories were present (Fig.1B(a-c)), but there was a higher proportion of  
15 cells in clusters possessing lower levels of ADAMTS-4 as reflected in the sharper lower IF intensity  
16 peak. There was no difference between ADAMTS-4 labelling for normal cells ( $P=0.9980$ ), cells with  
17 processes ( $P>0.9999$ ) or cells in a cluster between G0 and G1 ( $P=0.9744$ ; $[3(436)]$ ) suggesting  
18 morphological/clustering properties of the cells determined ADAMTS-4 levels and not cartilage  
19 grade. Although we have graded cartilage, our description of a small population of morphologically-  
20 abnormal chondrocytes in apparently G0 cartilage (Fig.1B(a)) raises an interesting question about  
21 cartilage grading. Chondrocyte death and clustering in histological sections are important criteria for  
22 cartilage grading however observing subtle changes in chondrocyte morphology requires different  
23 approaches such as those described here. We accept that a limitation is that the G0 cartilage studied  
24 here might have microscopic characteristics of very early cartilage degeneration and as such might  
25 not be 'truly' non-degenerate. Nevertheless we feel that the identification of aberrant chondrocytes  
26 provides an important insight into initial changes associated with cartilage degeneration.  
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50 CLSM and imaging identified marked heterogeneity in ADAMTS-4 levels which depended on  
51 chondrocyte shape and clustering. This approach assesses the responses of single *in situ* cells and  
52 revealed subtle and local properties of chondrocytes obscured with whole tissue samples.  
53 Morphologically-abnormal chondrocytes labelled more strongly for ADAMTS-4 compared to normal  
54 or clustered chondrocytes (Fig.2B(i)). As the chondrocyte population changed with cartilage  
55 degeneration (i.e. with more abnormal cells and clusters) this was reflected in a shift to left of the  
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2 distribution curve as there was a large number of cells in clusters with lower levels of ADAMTS-4  
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4 (Fig.2(B)(ii)). There was also heterogeneous labelling in each of the chondrocyte categories but there  
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6 was no *a priori* reason that cells in each category should behave identically. Examples showed  
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8 chondrocytes with no clear ADAMTS-4 labelling beside strongly-labelled cells, and even cells within  
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10 the same lacuna exhibiting varying levels of labelling (Fig.2A(iv)). This could be due to variations in  
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12 primary and/or secondary antibody penetration, however heterogeneity was observed in single  
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14 sections and images where all cells would be exposed to identical antibody levels. A more likely  
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16 explanation could be that access of factors controlling ADAMTS-4 production are variable and  
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18 dependent e.g. on local ECM permeability. While IL-1 $\beta$  upregulates ADAMTS-4 gene expression in  
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20 human chondrocytes and cartilage explants<sup>3</sup>, cell-associated levels of IL-1 $\beta$  are very different  
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22 between *in situ* human chondrocytes of varying morphology<sup>13</sup>. Thus IL-1 $\beta$  availability either from  
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24 intracellular metabolism or an inflammatory response, could be variable accounting for the  
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26 heterogeneity in ADAMTS-4 levels. The estimated  $t_{1/2}$  of ADAMTS-4 exocytosis is ~220mins<sup>15</sup> and it  
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28 is possible that there are varying levels of ADAMTS-4 depending on local IL-1 $\beta$  concentrations. We  
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30 also do not know the aggrecan levels around chondrocytes, however as they may be reduced in the  
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32 vicinity of morphologically-abnormal cells, this could lead to focal regions of mechanical weakness.  
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29 **Key words:** chondrocyte, osteoarthritis, ADAMTS-4.  
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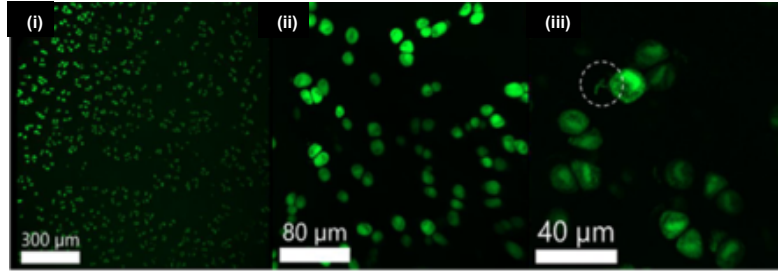
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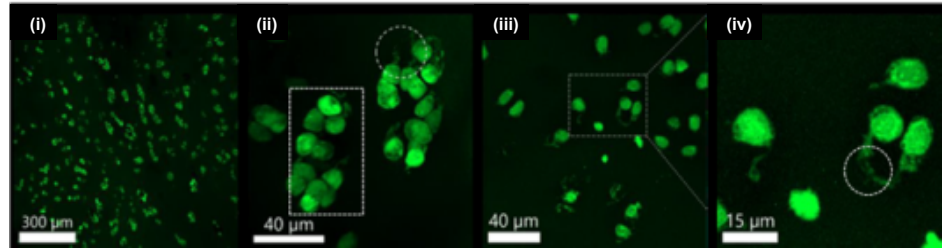
# Herren et al (2023) Figure 1.

(A)

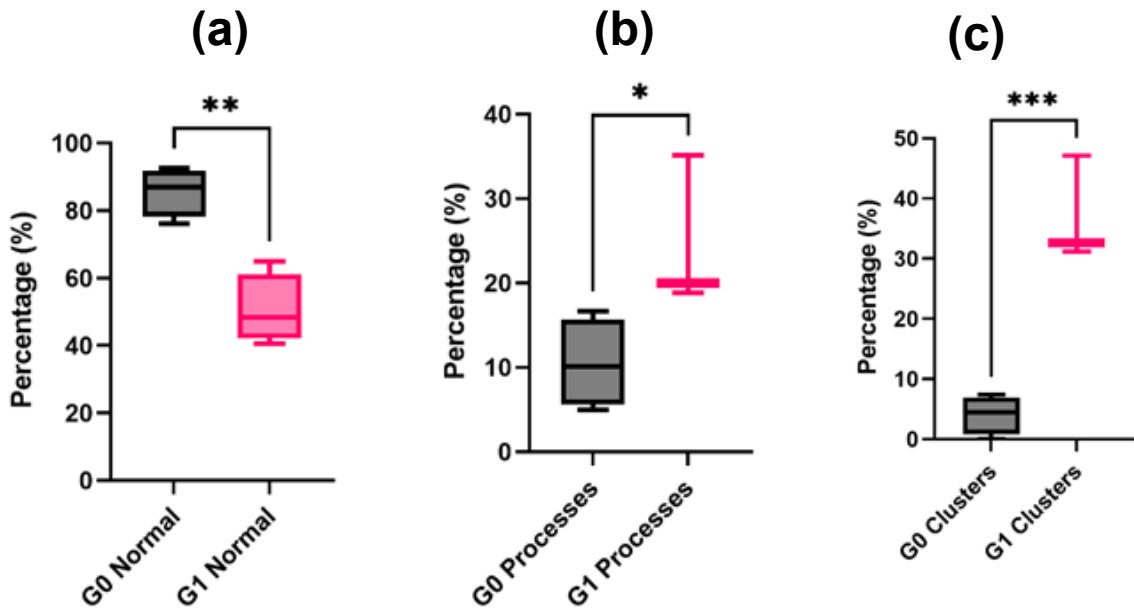
(a)  
(G0 cartilage)



(b)  
(G1 cartilage)

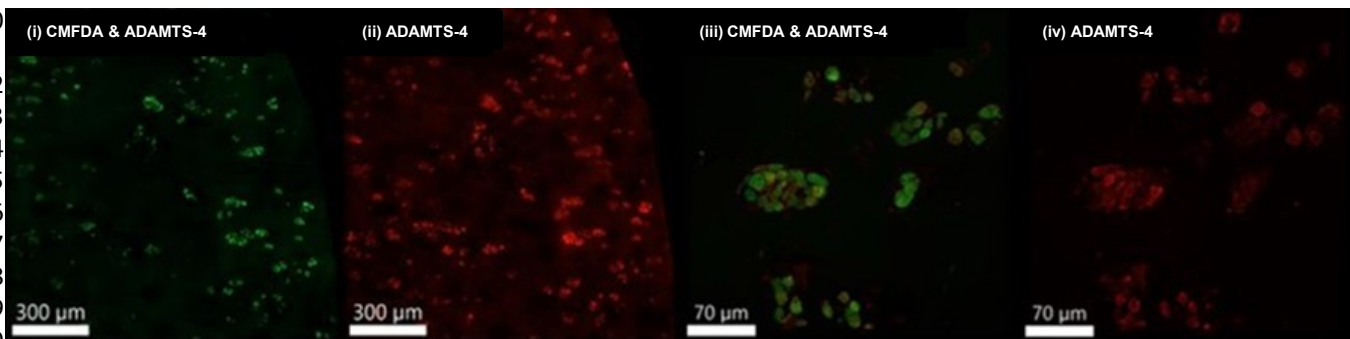


(B)



Herren et al (2023) Figure 2.

(A)



(B)

