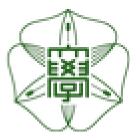

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1 **Metallothionein-3 is a clinical biomarker for tissue zinc levels in nasal mucosa.**

2

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12

13 Running title: MT3 is a biomarker for zinc in nasal mucosa.

14

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26

27

Metallothionein-3 is a clinical biomarker for tissue zinc levels in nasal mucosa.

ABSTRUCT

Objective: Recently, depleted tissue zinc levels were found in nasal mucosa from patients with chronic rhinosinusitis (CRS) in correlation with tissue eosinophilia, however, no clinical biomarkers for tissue zinc levels have been identified. Metallothionein-3 (MT3) is an intracellular zinc chelator and previous data showed MT3 mRNA levels to be reduced in CRS patients with nasal polyps (CRSwNP). In this study, we examined the correlation between MT3 expression and zinc levels in nasal mucosa and primary human nasal epithelial cells (HNECs) to investigate whether MT3 could be a clinical biomarker for tissue zinc levels.

Method: Tissue was harvested from 36 patients and mounted on tissue micro-array (TMA) slides. MT3 expression and tissue zinc fluorescence intensity were measured at different areas within the mucosa (surface epithelium and lamina propria) and compared between controls, CRSwNP and CRS without nasal polyps (CRSsNP) patients. MT3 mRNA and protein expression were examined in zinc-depleted HNECs by qPCR and immunofluorescence microscopy.

Results: MT3 expression in CRSwNP was significantly decreased in both surface epithelium ($p<0.001$ to controls) and lamina propria ($p=0.0491$ to controls). There was a significant positive correlation between tissue zinc levels and MT3 expression in nasal mucosa ($r=0.45$, $p=0.007$). In zinc-deplete HNECs, MT3 expression was significantly decreased at mRNA ($p=0.02$) and protein level ($p<0.01$). There was a significant positive correlation between tissue zinc levels and MT3 expression within individual HNECs ($r= 0.59$, $p<0.001$).

Conclusions: MT3 expression reflects intramucosal zinc levels in both nasal mucosa

and HNECs indicating MT3 could be used as a clinical biomarker for monitoring intracellular zinc levels in the nasal mucosa.

Key words: chronic rhinosinusitis, human nasal epithelial cells, zinquin, and nasal polyps

33 INTRODUCTION

34 Chronic rhinosinusitis (CRS) is defined as a sinonasal inflammation persisting
35 for more than 12 weeks, characterized by infiltration of inflammatory cells into the
36 nasal mucosa, tissue remodeling, and often accompanied by asthma(1, 2). CRS is
37 clinically classified into two subtypes depending on the presence or absence of nasal
38 polyps; CRS with nasal polyps (CRSwNP) and CRS without nasal polyps
39 (CRSsNP)(3).

40 Zinc is an essential trace element required for the normal functioning of many
41 organ systems including the immune system. Zinc deficiency decreases the function
42 of Th1 cells, but not that of Th2 cells and is associated with Th2 polarisation and
43 eosinophilic inflammation (4-6). Zinc depleted diet has been shown to promote
44 eosinophil infiltration into the lung in mice (7). Zinc depletion has been postulated to
45 be involved in the pathogenesis of bronchial asthma and atopic dermatitis(8-10).

46 Recently, depleted tissue zinc levels were reported in the nasal mucosa of
47 patients with CRS(11). Interestingly, whilst tissue zinc levels were decreased, there
48 was an increase in mucus zinc levels at sites of inflammation and no evidence of
49 systemic zinc depletion (11, 12). Mucosal zinc depletion is thought to be involved in
50 multiple aspects of the pathophysiology of CRS and is more severe in CRSwNP than
51 CRSsNP (11, 12). Reduced zinc levels impair epithelial barrier function at the level of
52 the nasal mucosa and promote pro-inflammatory cytokines expression(11). Mucosal
53 zinc levels are inversely correlated with eosinophil infiltration and affect tissue
54 remodeling, which concerns a cycle of deposition and removal of extracellular matrix
55 (ECM) proteins(12). Tissue eosinophil infiltration is seen in up to 70% of CRSwNP
56 patients and those patients often have extensive disease and poor prognosis compared
57 to non-eosinophilic CRS patients(13, 14).

58 Despite evidence of altered homeostasis of zinc in eosinophilic CRSwNP
59 patients and the role of zinc in the pathophysiology of CRS, there are no methods to
60 evaluate mucosal zinc levels in clinical practice. Serum zinc levels and zinc
61 transporter genes in nasal mucosa are not useful biomarkers, because there were no
62 significant differences in serum zinc levels and zinc transporter genes between
63 healthy controls and CRS patients(11, 12).

64 Metallothionein-3 (MT3) is a zinc chelator gene, the expression of which is
65 regulated by intracellular zinc levels. Our previous data showed MT3 mRNA was
66 significantly reduced in CRSwNP compared to controls (11). These findings indicate
67 that MT3 expression could be a new biomarker for mucosal zinc levels. However,
68 there are no reports on MT3 expression in nasal mucosa from CRS patients in relation
69 to mucosal zinc levels. It is also unknown whether MT3 expression reflects
70 intracellular zinc levels in primary human nasal epithelial cells (HNECs).

71 In this study, we examined the relationship between MT3 expression and tissue zinc
72 levels using a tissue micro array (TMA). We also investigated the effect of zinc
73 depletion on MT3 expression in HNECs using zinc-depleted medium.

74

75

76 MATERIALS AND METHODS

77 *Patients and tissue collection*

78 The study was approved by The Queen Elizabeth Hospital Human Research
79 Ethics Committee (reference HREC/15/TQEH/132). Written informed consent was
80 obtained from study participants prior to tissue or cell collection. Nasal mucosa was
81 taken from those patients undergoing endoscopic sinus surgery (ESS) for CRS or
82 skull base tumor resections. Donors were divided into control patients (patients with
83 skull base tumor), and CRSsNP or CRSwNP, depending on the absence or presence
84 of nasal polyps respectively, as defined by the European Position Paper(3). Ethmoidal
85 mucosa was used in control and CRSsNP patients and nasal polyps from middle
86 meatus were used for CRSwNP patients. Control patients were only included in the
87 absence of radiographic or endoscopic evidence of CRS. Patients with
88 immunosuppression and treatments with oral antibiotics or corticosteroids in the week
89 prior to study initiation were excluded. The sample pathologically diagnosed as
90 respiratory epithelial adenomatoid hamartoma (REAH) or nasal tumor were excluded.
91

92 *Primary human nasal epithelial cell cultures*

93 Primary human nasal epithelial cells (HNECs) were taken from the nasal
94 mucosa of donors in a method as previously described(15-17). The cells were
95 suspended in Bronchial Epithelial Growth Medium (BEGM, CC-3170, Lonza,
96 Walkersville, MD, USA), supplemented with 2% Ultroser G (Pall Corporation, Port
97 Washington, NY, USA). Monocytes were depleted from the cell suspension using
98 anti-CD68 antibody (Dako, Glostrup, Denmark) coated cell culture dishes. HNECs
99 were incubated in 37°C, humidified, 5% CO_2 in collagen-coated flasks (Thermo
100 Scientific, Walthman, MA, USA), until passage 2. The cells were confirmed of being

101 epithelial lineage by reactivity to PAN-cytokeratin and CD45 antibodies, and by
102 morphological examination with Diff-Quik analysis by qualified cytologists (IMVS
103 Cytology Department, The Queen Elizabeth Hospital, Adelaide, Australia).
104
105 *Zinc depleted media*
106 5 grams of analytical grade Chelex 100 chelating ion-exchange resin (C7901,
107 Sigma, Saint Louis, MO, USA) in two separate 50 ml falcon tubes was washed twice
108 with Milli-Q water for 10 minutes. 50 ml of BEGM was added into the first tube and
109 incubated for 2 hours on a rotator at room temperature (RT), following transfer into
110 the other tube. After incubation overnight on a rotator at 4°C, the Chelex beads were
111 discarded and the media was collected. The depleted molecules except zinc were
112 added to the media ($CaCl_2$=22.19mg, $Fe(NO_3)_3$=0.00014mg, $FeSO_4$=0.022mg,
113 $MgSO_4$=2.4mg, $MgCl_2$=4.28mg). The pH of the media was adjusted to original
114 BEGM.
115
116 *RNA extraction, Reverse transcription and qPCR.*
117 HNECs were incubated in 6-well plates in normal and zinc deplete BEGM for
118 48 hours. Cells were collected and washed in phosphate buffered saline (PBS),
119 followed by RNA extraction using the RNeasy Mini Kit according to the
120 manufacturer's instructions (QIAGEN Pty Ltd., Australia). An on-column DNAse
121 treatment was performed with an RNase-Free DNase Set (QIAGEN Pty Ltd.,
122 Australia). Extracted RNA was quantified using the Nanodrop 1000
123 spectrophotometer (Thermo Fisher Scientific, Franklin, MA, USA). RNA was reverse
124 transcribed into cDNA using Quantitect Reverse Transcription kit (Qiagen, Hilden,
125 Germany) with a MyCycler Thermal Cycler (BioRad Laboratories Inc., Gladesville,

126 Australia). The resulting cDNA was subjected to qPCR with TAQman primer/probe

127 sets for each target gene, Taqman Universal Master Mix II (Thermo Fisher Scientific,

128 Scoresby, Australia) and nuclease-free water. The average threshold cycle (Ct) was

129 determined from three independent experiments and the level of gene expression

130 relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined

131 with the comparative CT method. Taqman Gene Assays used for gene expression

132 analysis were: Hs00359394_g1 (MT3) and Hs02758991 (GAPDH).

133

134 *Tissue Microarray*

135 The analyses were conducted with the approval of the local Human Research

136 Ethics Committee. Archival formalin-fixed paraffin-embedded sinus tissue blocks

137 collected from the patients who underwent sinonasal surgery were used to construct

138 the tissue microarray (TMA) for immunofluorescence. To guide the location of tissue

139 cores, a 4 µm section was sliced from each tissue block and stained using

140 Haematoxylin and Eosin (H&E). The tissue cores were punched using a 2 mm

141 disposable biopsy punch (Kai medical, Kai Europe GmbH, Solingen, Germany) and

142 manually put into Quick-RayTM paraffin recipient block 2 mm × 60 wells (IHC

143 World, Woodstock, MD, USA).

144

145 *Immunofluorescence*

146 Tissue samples were cut in 4 µm sections from the TMA block. The slice was

147 rehydrated, followed by antigen retrieval by submerging the slides in sodium citrate

148 buffer (10 mM sodium citrate buffer, pH 6.8) and incubating in a 1100W microwave

149 for 10 minutes. After cooling, sections were incubated with Serum Free Blocker (SFB,

150 Dako, Glostrup, Denmark) for 60 minutes at RT, followed by permeabilization with

151 1% SDS in PBS. The sections were blocked with SFB for 60 minutes at RT. Rabbit
152 polyclonal metallothionein 3 (MT3) antibody (A27974, Sigma-Aldrich, St Louis, MO,
153 USA), diluted to 4 μg/mL in Tris-Buffered Saline-0.5% Tween (TBST), was added
154 and incubated overnight at 4 °C followed by washing with TBST to remove excess
155 primary antibody. 2 μg/mL Cy3-conjugated donkey anti-rabbit antibody (Jackson
156 ImmunoResearch Labs Inc, West Grove, PA, USA) was then added and cells were
157 incubated for 1 hour at RT, followed by washing in TBST three times. 25 μmol/L of
158 zinquin ethylester (Sigma-Aldrich, St. Louis, MO, USA) was added for 30 minutes at
159 37°C. The slides were washed, mounted with fluorescent mounting medium (Dako,
160 Glostrup, Denmark) and visualized by using a LSM700 Confocal Laser Scanning
161 Microscope (Zeiss Microscopy, Oberkochen, Germany) using the 20x objective.
162 Excitation and emission values for MT-3 and zinquin were 555/570 nm and 405/460
163 nm, respectively. All images were acquired with identical microscopy parameters and
164 taken sequentially to minimise cross-talk between fluorophores. Processing was
165 performed using ZEN Imaging Software (Carl Zeiss AG, Oberkochen, Germany).
166 The threshold of images was set for each channel to subtract autofluorescence and
167 background fluorescence due to unspecific binding of antibody. For each specimen, at
168 least 3 areas of the epithelium and the lamina propria were randomly selected as a
169 region of interest (ROI) as described(18-20), then quantification of zinquin intensity
170 was performed at the area. Quantification of MT-3 fluorescence intensity was
171 acquired within the same ROI where zinquin intensity was measured (Supplemental
172 Figure 1). Results are expressed as mean arbitrary fluorescence units, provided by the
173 ZEN imaging software.
174 For immunofluorescence of HNECs, the cells were fixed with 2.5% formalin
175 in PBS for 10 minutes followed by washing with TBST four times. The cells were

176 permeabilized with 1% SDS in PBS and blocked with SFB. The following process
177 was identical as described above.
178
179 *Statistical Analysis*
180 All data was expressed as mean ± Standard Deviation (SD). The intensity of
181 MT3 fluorescence was compared with Kruskal-Wallis test. When 3 or more groups
182 were compared, one-way Analysis Of Variance (ANOVA) followed by Tukey's test
183 was used to analyze differences among the groups. The Pearson correlation test was
184 used to determine the correlation among MT3 and zinquin intensity. P-values of
185 <0.05 were considered statistically significant. All the analyses were performed by
186 using the JMP® 11 (SAS Institute Inc., Cary, NC, USA).
187

188 RESULTS

189 ***MT3 expression is decreased in the mucosa of CRSwNP patients.***

190 First, we examined MT3 expression and zinquin fluorescence within the
191 mucosa using immunofluorescence assays on a TMA slide that included tissue cores
192 from 8 controls, 15 CRSsNP, and 13 CRSwNP. MT3 and zinquin fluorescence
193 intensity were measured at different areas within the mucosa (surface epithelium and
194 lamina propria) and compared amongst patient groups (Figure 1A). MT3 intensity in
195 CRSwNP tissue was significantly decreased in both surface epithelium (p=0.0006 to
196 controls, Figure 1B) and lamina propria (p=0.0491 to controls, Figure 1C). MT3
197 intensity in CRSsNP tissue was also significantly decreased in the surface epithelium
198 compared to controls (p=0.003, Figure 1B).

199

200 ***MT3 expression positively correlated with zinc levels in nasal mucosa.***

201 Next, we determined the relation of labile zinc levels with the expression of
202 MT3 using immunofluorescence analysis of the TMA. There was a significant
203 positive correlation between MT3 and zinquin fluorescence intensity in nasal mucosa
204 (r=0.45, p=0.007, Figure 2).

205

206 ***MT3 expression correlates with zinc levels in nasal epithelial cells.***

207 Next, we investigated the effect of zinc depletion of HNECs on MT3
208 expression. We established zinc deplete cells by culturing HNECs in zinc deplete
209 medium for 48 hours as previously reported(11, 12). Compared to control cells, in
210 zinc-deplete cells, MT3 fluorescence intensity was significantly decreased (control
211 57.83±10.83 vs zinc-deplete cells 36.68±4.51, p<0.01, Figure 3A and 3B). MT3
212 mRNA in zinc-depleted cells was also significantly decreased (0.45±0.26 fold change,

213 p=0.02, Figure 3C). There was a significant positive correlation between zinquin and

214 MT3 fluorescence intensity within individual cells ($r= 0.59$, $p<0.001$) (Figure 3D).

215

216 DISCUSSION

217 This study showed that MT3 fluorescence intensity and mRNA expression
218 were significantly decreased in zinc-depleted HNECs in relation to zinc levels. In
219 addition, MT3 expression and labile zinc levels were significantly decreased in the
220 mucosa of CRSwNP patients, corresponding to previous reports showing zinc
221 depletion with decreased MT3 mRNA expression in CRSwNP(11). These results
222 suggest that MT3 mRNA and protein expression could be a new biomarker for tissue
223 zinc levels in nasal mucosa.

224 Zinc is an essential micronutrient and has a critical role in physiologic,
225 enzymatic and structural functions(21). Consequently, altered zinc homeostasis
226 affects multiple organs and systems and causes impaired immune function. In fact,
227 zinc depletion has been reported in many diseases, including rheumatoid arthritis(22),
228 hypertension(23), neurodegenerative diseases(24), Inflammatory Bowel Disease
229 (IBD)(25), type 2 diabetes mellitus(26, 27), atopic dermatitis(8), bronchial asthma(28,
230 29), and chronic rhinosinusitis (CRS)(10-12, 30).

231 Recently, altered zinc homeostasis has been thought to be involved in multiple
232 aspects of the pathophysiology of CRS, especially in CRSwNP patients(10). This
233 includes impaired epithelial barrier function, eosinophil infiltration and tissue
234 remodeling(11, 12). CRSwNP patients have been shown to present with lower
235 mucosal zinc levels in association with a reduced expression of the tight junction
236 protein Zonula Occludens-1 (ZO-1) compared to controls(11). This finding is
237 supported by *in-vitro* results showing that zinc-depleted HNECs formed leaky
238 mucosal barriers (11).

239 Depleted zinc levels also affect immune responses in the nasal mucosa and
240 have been shown to promote pro-inflammatory cytokine production in HNECs(12).

241 Also, significant negative correlations between tissue zinc levels and eosinophil
242 infiltration into the nasal mucosa of CRS patients have been demonstrated, in line
243 with previous findings of Th2 polarisation and eosinophilia in the context of zinc
244 deficiency(4-6). Tissue zinc levels are also correlated with collagen abundance in
245 nasal mucosa and zinc depleted-fibroblasts synthesized less collagen compared to
246 control cells. Considering that eosinophil infiltration and decreased collagen
247 expression in nasal mucosa are hallmarks of CRSwNP, it has been postulated that
248 depleted zinc levels might play a role in the formation of nasal polyps(12). Moreover,
249 since tissue eosinophilia is often seen in CRS patients in relation to severity of disease,
250 mucosal zinc levels and MT3 expression might also relate to disease severity and
251 outcomes after surgery in those patients(13, 14). More research into the potential
252 relationship between disease severity and mucosal zinc levels is needed to evaluate
253 this hypothesis.
254
255 So far, there have been no standard methods to examine mucosal zinc levels in
256 daily practice. Zinquin, the ultraviolet-excitable zinc-specific fluorophore, has been
257 used to visualize tissue zinc in airways, but only for research purposes. It requires
258 special equipment such as confocal laser microscopy and advanced imaging
259 techniques and is a semi-quantitative technique to evaluate labile zinc levels.
260 It has been well documented that MT3 expression is strictly regulated by
261 intracellular zinc levels through metal response elements (MRE) present in the
262 promoter region of metallothionein genes. Intracellular zinc activates MRE-binding
263 transcription factor-1 (MTF-1). The activated MTF-1 binds to MRE and promotes
264 MT3 expression (Figure 4)(31-33). In contrast, depletion of intracellular zinc levels
265 suppresses the activity of MTF-1, resulting in decreased MT3 expression. These

266 known mechanisms of transcriptional regulation of MT3 expression by intracellular
267 zinc levels support the potential for MT3 mRNA expression to be used as a biomarker
268 for tissue zinc levels in nasal mucosa. Interestingly, this theoretical framework of
269 MT3 gene regulation matches our findings of reduced MT3 mRNA and protein levels
270 in relation to reduced zinc levels in HNECs as well as results of our previous study
271 showing a reduced MT3 mRNA expression in CRSwNP patients in relation to
272 depleted tissue zinc levels(11).

273 From a clinical viewpoint, it could be more applicable to examine MT3
274 mRNA than MT3 or zinquin fluorescence intensity, as mRNA is more readily
275 examined with qPCR and does not require special equipment such as confocal laser
276 microscopy and advanced imaging techniques. Also, analysis of MT3 mRNA allows
277 for more precise quantitation as compared to semi-quantitative methods relying on
278 fluorescence. In general, immunofluorescence provides MT3 or zinquin intensity as
279 relative values to other samples put on the same slide. In this research, we constructed
280 the TMA slide containing 36 samples so that fluorescence intensity of the different
281 samples could be compared under the same condition. Whereas immunofluorescence
282 analysis using TMA slides therefore more accurately define relative protein/cellular
283 content, the use of such techniques is not applicable in clinical practice. In contrast,
284 intramucosal zinc levels could be more readily predicted by MT3 mRNA levels
285 allowing more accurate comparison among different samples measured at different
286 times and places.

287

288 It is unknown whether reduced MT3 expression plays a role in the
289 pathophysiology of CRSwNP. MT3 has been shown to protect against airway
290 inflammation induced by Ovalbumin(34), indicating a potential beneficial role for

MT3 in allergic airway disease. MT3 might have a protective role for airway inflammation in the nasal cavity as well. Further study will be necessary to unravel the exact role of MT3 in the pathogenesis of CRS.

CONCLUSION

MT3 expression reflects intramucosal zinc levels in both nasal mucosa and HNECs indicating MT3 could be used as a clinical biomarker for monitoring intracellular zinc levels in the nasal mucosa.

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306

307 AUTHORSHIP CONTRIBUTION

308 MS, YN, and SV designed the experiments. AJP, AH, and PJW supervised the
309 project. MS and MR performed most of the experiments and compiled the data. CC
310 and KO performed sample preparation. MS, MR, KO, and SV wrote the manuscript.
311 All authors provided feedback on the manuscript.

312

313 DISCLOSURE STATEMENT

314 None of the authors have any conflicts of interest or financial disclosures that
315 are relevant to this study.

316

317

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430

431 FIGURE LEGENDS

432 ***Figure 1. MT3 expression is decreased in the surface epithelium and lamina***

433 ***propria in CRSwNP.***

434 A. Immunofluorescence of representative control, CRSsNP and CRSwNP patients

435 using Zinquin (blue staining) and MT3 antibody (red staining). B and C. MT3

436 fluorescence intensity between the different patient groups was compared in the

437 surface epithelium (B) and the lamina propria (C) in nasal mucosa. *$p<0.05$,

438 Wilcoxon test.

439

440 ***Figure 2. MT3 expression positively correlated with zinc levels in nasal mucosa.***

441 Pearson's correlation coefficient between MT3 and Zinquin fluorescence in the lamina

442 propria of nasal mucosa is shown in a scatter diagram with 99% (red), 95% (green),

443 90% (blue), and 50% (purple) probability eclipses, respectively. Skyblue, orange, and

444 gray points stand for CRSwNP, CRSsNP and control, respectively.

445

446 ***Figure 3. MT3 expression correlates with zinc levels in nasal epithelial cells.***

447 A-D. HNECs were incubated for 48 hours in zinc depleted (ZD) or control media

448 followed by immunofluorescence using MT3 antibodies (A, B, and D) and by qPCR

449 using MT3 primers (C). Data are means ± standard deviation (s.d.) of values from at

450 least three independent experiments. P values for indicated comparisons were

451 determined by t-test. *$p<0.05$. D. Zinquin and MT3 fluorescence intensity per HNEC

452 was shown in a scatter diagram. The correlations between the zinquin and MT3

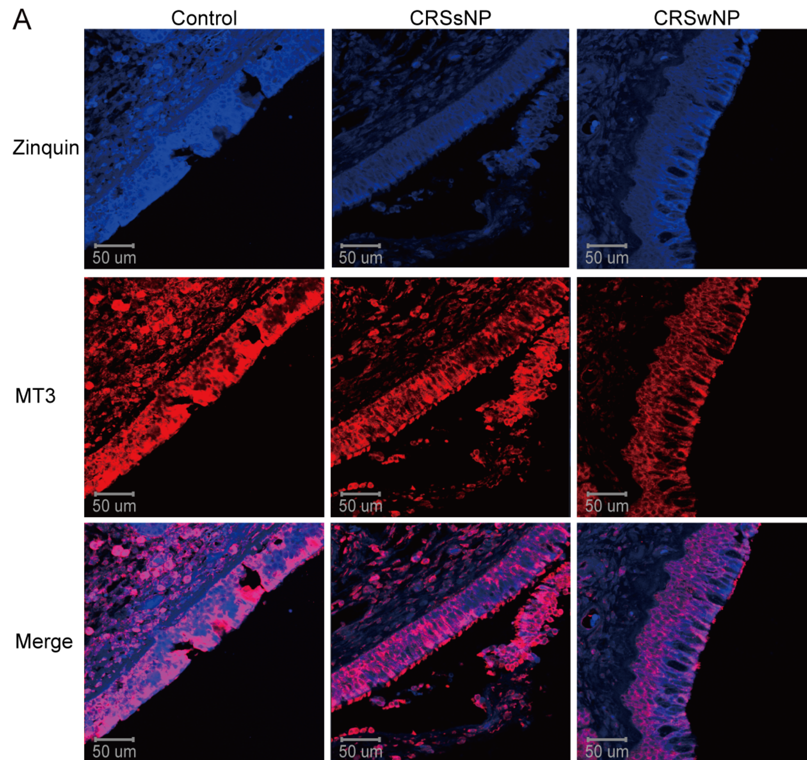
453 fluorescence intensity were tested using Pearson's correlation coefficient.

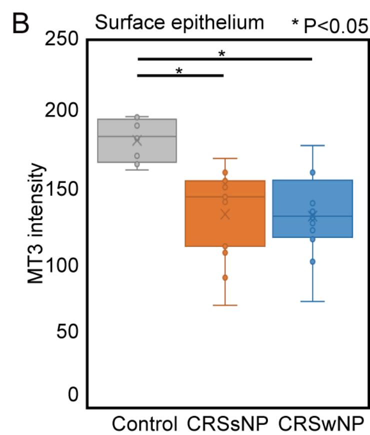
454

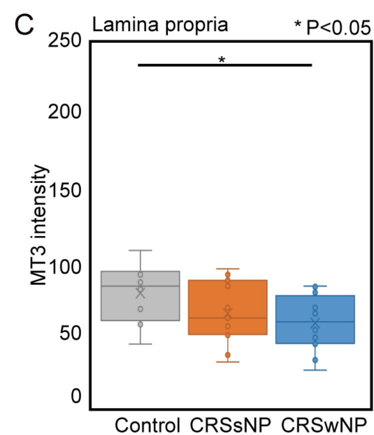
455 ***Figure 4. MT3 expression correlates with intracellular zinc levels.***

456 Free intracellular zinc activates metal response elements (MRE)-binding transcription
457 factor-1 (MTF-1). MT3 expression is regulated by binding of activated MTF-1 to
458 MRE located in the promotor region of MT-3 promoting MT3 mRNA expression (31-
459 33). Depleted intracellular zinc fails to activate MTF-1 resulting in decreased MT3
460 expression.
461
462 ***Supplementary Figure 1. MT3 expression correlates with intracellular zinc levels.***
463 For each specimen, at least 3 areas of the epithelium and the lamina propria were
464 randomly selected as a region of interest (ROI), then quantification of zinquin and
465 MT-3 fluorescence intensity was performed at the area.
466
467

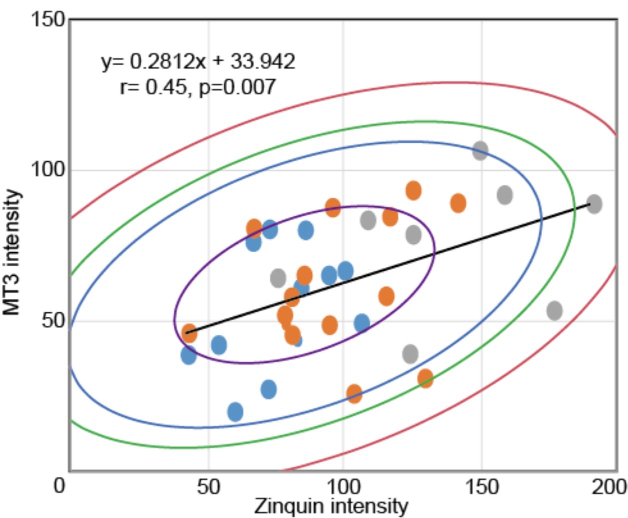
M Suzuki, MT3 figure 1



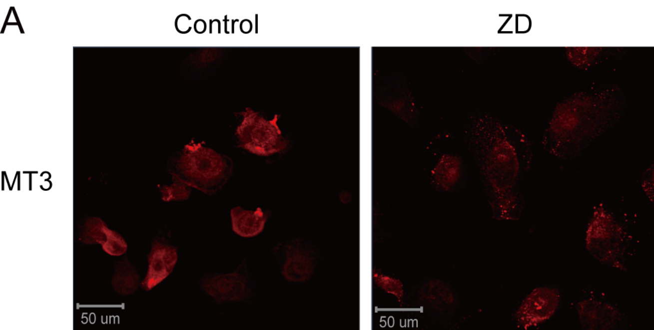


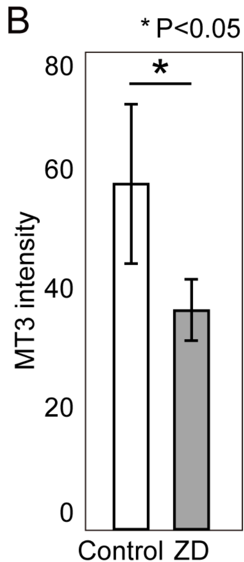


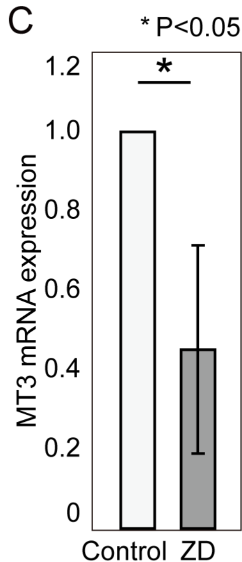
M Suzuki, MT3 figure 2

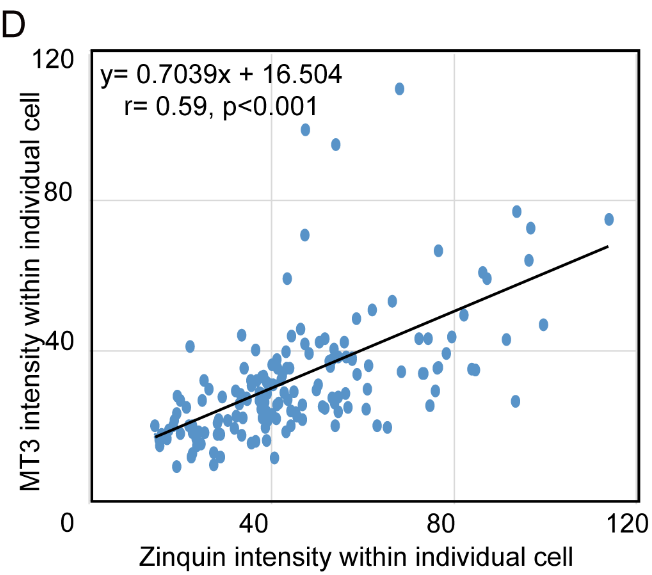


M Suzuki, MT3 figure 3









M Suzuki, MT3 figure 4