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Reduced expression of angiotensin II and angiotensin receptor type 1 and type 2 in resistance arteries from nasal lesions in granulomatosis with polyangiitis (Wegener's)

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KEY WORDS: AT₁ receptor; AT₂ receptor; Angiotensin II; immunohistochemistry; nasal biopsy, granulomatosis with polyangiitis (Wegener's), vascular

Objective Angiotensin II (ANGII) is involved in vessel inflammation and is important in the development of cardiovascular disorders such as atherosclerosis. During active disease, patients with granulomatosis with polyangiitis (Wegener's) (GPA) have accelerated atherosclerosis and ANGII inhibitors are recommended to these patients to reduce atherosclerosis. We assessed the hypothesis that the expression of ANGII and its receptors in arteries in granulomatous lesions change in GPA.

Methods ANGII and angiotensin receptors were quantified in vessels from granulomatous lesions from patients with GPA using immunohistochemistry. Anti-Angiotensin II, AT₁ and AT₂ antibodies were applied on formalin fixed and paraffin embedded biopsies from nasal mucous membranes from eight patients with GPA and eight controls.

Results ANGII expression was localized to the endothelial cells (EC) in arteries and sparsely to vascular smooth muscle cells (VSMC) in nasal biopsies. AT₁R staining was solid and located in the VSMC in the medial layer of the control arteries. AT₂R immunostaining was only located in EC and the immunostaining was faint. Patients with GPA showed marked down regulation of positively immunostained EC for ANGII or AT₂R, reduced number of AT₁R in VSMC. ANGII, AT₁R and AT₂R staining was persistent on infiltrating leucocytes.

Conclusions These results suggest down regulation of the Angiotensin system in arteries in granulomatous nasal lesions in GPA. Inhibition of the angiotensin system may prove less efficient in inhibiting the vascular inflammation process in GPA.

Introduction

Granulomatosis with polyangiitis (Wegener's) (GPA) is a vascular disease characterized by granulomatous inflammation and systemic vasculitis predominantly in small vessels, commonly found in the respiratory tract[1]. Patients usually present local symptoms from the upper respiratory tract such as nasal crusting, sinusitis or nasal obstruction. ANCA prompt tissue damage via interactions with primed neutrophils and endothelial cells (EC) inducing diffuse endothelial dysfunction as reported in GPA [2].

Angiotensin II (ANGII) is implicated in EC dysfunction and is the main effector of the renin–angiotensin–aldosterone system (RAAS) with direct vascular effects. Apart from circulating ANGII there is also locally produced ANGII in the vascular endothelium. RAAS plays a major part in vasoconstriction [3, 4], in hypertension and in tissue injury, and is regulated independently of circulating ANGII [5]. In vascular smooth muscle cells (VSMC), local ANGII may be more important than circulating ANGII in vascular disorders[6]. ANGII blockage via angiotensin converting enzyme inhibitors (ACEI) or receptor inhibition is recommended for reduction of cardiovascular risk not only in atherosclerosis, but GPA as well. In this study, we have examined local ANGII expression, AT₁R and AT₂R in arteries from nasal biopsies with granulomatosis in patient with GPA using immunohistochemistry.

MATERIALS AND METHODS

Patients and Tissue collection

Nasal biopsy specimens from 16 patients were retrieved from archive at the Department of Pathology, (Helsingborg Hospital, Sweden). The original pathology reports were reviewed to verify the diagnosis GPA. The diagnosis was based on the American College of

Rheumatology 1990 criteria for the classification of GPA [7]. Biopsies for nasal polyposis served as controls (n=8). The samples were handled in accordance with permit obtained from the regional Human Ethics Committee.

Immunohistochemistry

Sections were prepared as previously described [8]. Incubated 1 h at room temperature with monoclonal mouse anti smooth muscle actin antibodies (Sc-53015, SC = *Santa Cruz Biotech., Inc. CA, USA*) and subsequently donkey anti-mouse Texas Red (Jackson ImmunoResearch, West Grove, PA) for 1 h in room temperature followed by double immunostaining using rabbit polyclonal anti ANGII antibodies (NBP1-30027, Novus Biologicals, LLC, Littleton, CO, USA), AT₁R (Sc-1173) or AT₂R (Sc-9034). Fluorescein isothiocyanate FITC (Cayman Chemical, Ann Arbor, MI, USA) or Alexa 488 (Invitrogen, La Jolla, CA, USA) antibodies allowed for protein visualization. Vectashield medium containing 4', 6-diamidino-2-phenylindole (DAPI) stained the cell (Vectashield, Vector Laboratories Inc, Burlingame CA, U.S.A.). Details on dilutions and antibody specificity are given on the company's homepage. The microscope (Olympus BX 60, Japan) was set at appropriate wavelengths and antigen expression was scored (arbitrary scale) in a blinded manner on a scale from 0 to 4 (0=no staining, 1=<25% antigen expressing cells, 2=25–50% antigen expressing cells, 3=50-75% antigen expressing cells and 4=>75% antigen expressing cells). Digital images were merged. Exclusion of primary antibodies lead to inappropriate binding of the secondary antibody and served as negative control.

Calculations and Statistics

The data were evaluated in a graded manner as non-parametric values and presented as the median and interquartile range. Exact values were presented counting the positive DAPI stained cells expressing immunostaining and presented with average values as parametric data. Group mean values were compared by non-parametric Mann-Whitney U test and average staining percentage as student t-test. Significance was defined as $P < 0.001$ (***) . Values are presented as median or means \pm standard error of the mean.

Results

Clinical and Hematoxylin-Eosin Staining

All subjects with GPA presented with symptoms from the respiratory tract at the time of biopsy. Foremost symptoms involved the nasal cavity (n=7) with nasal obstruction, purulent discharge or nasal crusting as well as otitis media. All patients had positive ANCA in conjunction with biopsy. The absence of granulomatous lesions in control samples from patients with nasal polyposis led to the classification of the sample as negative for GPA. All GPA patients had histopathology positive for granulomas and vasculitis (n=8) with inflammatory cells, including lymphocytes, histocytes and multinucleated giant cells (n=6). Nasal biopsies from eight patients with GPA were compared with eight patients with clinical nasal polyposis.

ANGII Immunostaining

Immunostaining for ANGII was observed in the EC layer (Fig 1A and 1B) in arteries from control patients scoring +3 (average $69\% \pm 4\%$), all controls had positive staining (n=8). In the EC layer of the arteries from patients with GPA, there was no positive immunostaining in 38% of the patients (n=3). The median score was +1 (average $18,8\% \pm 6,3$). Weak immunostaining of ANGII was observed in the smooth muscle cells in the vessel wall

(Fig1A). Intra and extra arterial lymphocytes stained positively for ANGII both in control vessels and vessels with GPA.

AT₁R Immunostaining

Immunostaining for AT₁R was observed in the smooth muscle cell layer (n=8) (Fig 2A) and localized to the cytoplasm. The VSMC layer of the arteries from patients with GPA also stained positive for AT₁R (Fig 2A) but the amount of positively stained VSMC differed markedly between the subjects and 38% (n=3) had no immunostaining. The GPA patients had a median score of +1 positive immunostaining (average $15,6\% \pm 4,5$) in the VSMC. Control patients had a median staining score of +4 (average $94\% \pm 15$) ($p < 0.001$) (Fig 2B). No immunostaining of AT₁R was detected in the EC layer in arteries from control patients or patients with GPA. In the granulomatous lesions from patients with GPA, AT₁R staining was observed in the lymphocytes (Fig 2A).

AT₂R Immunostaining

Immunostaining for AT₂R was primarily located in the EC layer (Fig 3A) (100%, n=8). Weak immunostaining was limited to the luminal part of the EC in healthy control subjects 100% (n=8). AT₂R expression in the EC was only visualized in 38% (n=3) even with intact endothelium in patients with GPA. The GPA patients had a median score of 0 positive immunostaining (average $9,4\% \pm 4,5$) in the VSMC. Control patients had a median staining score of +3 (average $69\% \pm 3,5$) ($p < 0.001$) (Fig 3B). Lymphocytes stained positively for AT₂R in GPA and control patients.

Discussion

Major findings

The major findings in this study were a general reduction of ANGII immunostaining in the EC, AT₁R downregulation in the VSMC together with reduced AT₂R expression in the ECs in GPA subjects. ANGII, AT₁R and AT₂R immunostaining in inflammatory cells was vast. ANGII receptors mediate the biologic effects via the AT₁R and AT₂R; these have been recognised and cloned in man[9]. AT₁Rs are located on VSMC, while AT₂Rs are found on EC.

Alternative pathways probably reduced AT₁R expression in VSMC. Different growth factors, cytokines and hormones can either up or down regulate the AT₁R expression[10] including C reactive protein (CRP)[11, 12], exaggerating the pathological effects of AT₁Rs.

The precise mechanisms prompting down regulation of ANGII and AT₁R expression in patients with GPA cannot be deduced from this study. Apart from systemic ANGII, inflammatory mediators involved in the intense inflammatory reaction taking place in the granulomatous lesions may have contributed altering the AT₁R and AT₂R expression. Various molecules in the granuloma may modulate the AT₁R expression in VSMC. Animal models as well as in vitro studies in humans have revealed that tumor necrosis factor- α (TNF- α) is involved in GPA [13]. Interleukin-1 α (IL-1 α), but not TNF- α or interferon- γ (IFN- γ), increases ANGII binding and AT₁mRNA levels, whereas the combination of IL-1 α , TNF- α , and IFN- γ decreases ANGII binding to VSMC [11]. In situations of intense systemic inflammation such as sepsis the combination of TNF- α and other cytokines [10] contributes to a decrease in AT₁R expression.

Our result shows a general downregulation of ANGII and the receptor subtypes. This down regulation of the angiotensin receptors were seen in VSMC or in the EC depending on the subtype of angiotensin receptor. Also ANGII is down regulated in the ECs in granulomatous lesions in GPA. The mechanisms altering ANGII or angiotensin receptors in vessels in GPA lesions cannot be concluded from the present study and the nature of this retrospective study did not allow for investigation of healthy vessels in GPA.

Conclusions

ANGII, AT₁R and AT₂R are expressed in the nasal vessel wall. A variety of cells, including glandular and nasal mucus membrane epithelium and infiltrating leucocytes also express the angiotensin receptors. Upon granulomatous inflammation as in GPA, a substantial reduction of AT₁R and AT₂R expression is noted in vessels with accompanying ANGII reduction. ANGII receptor inhibitors might prove less useful in GPA.

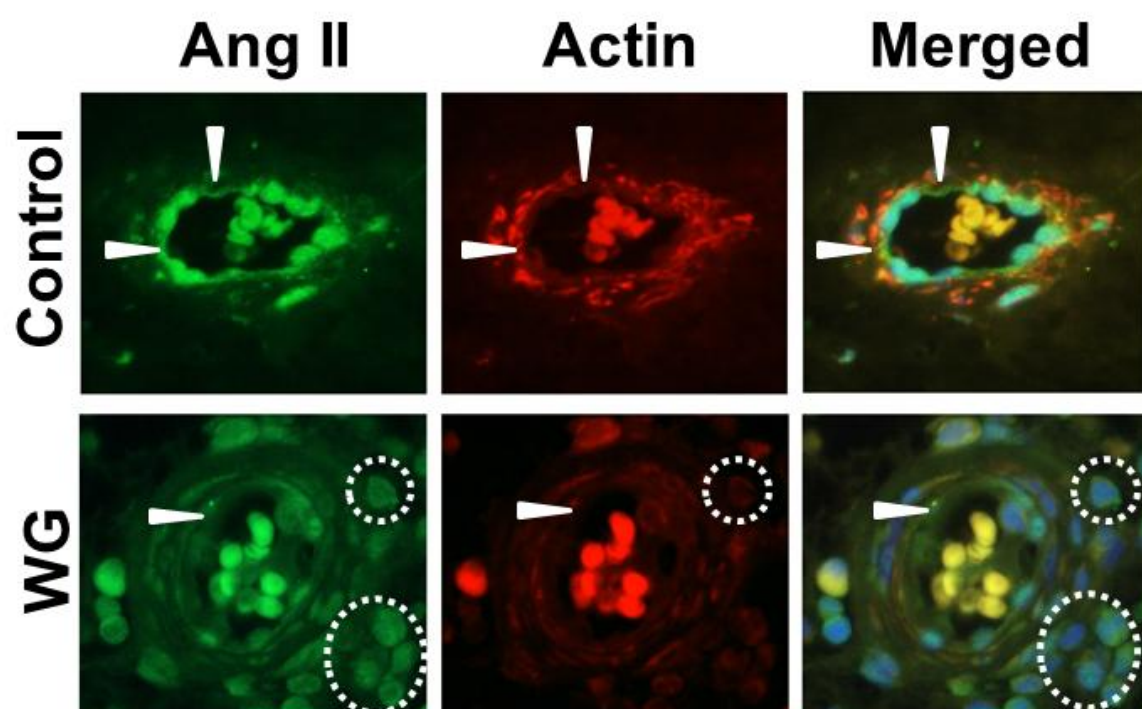
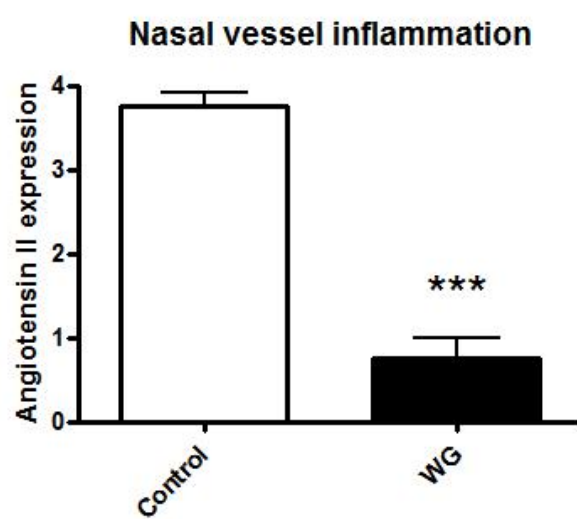
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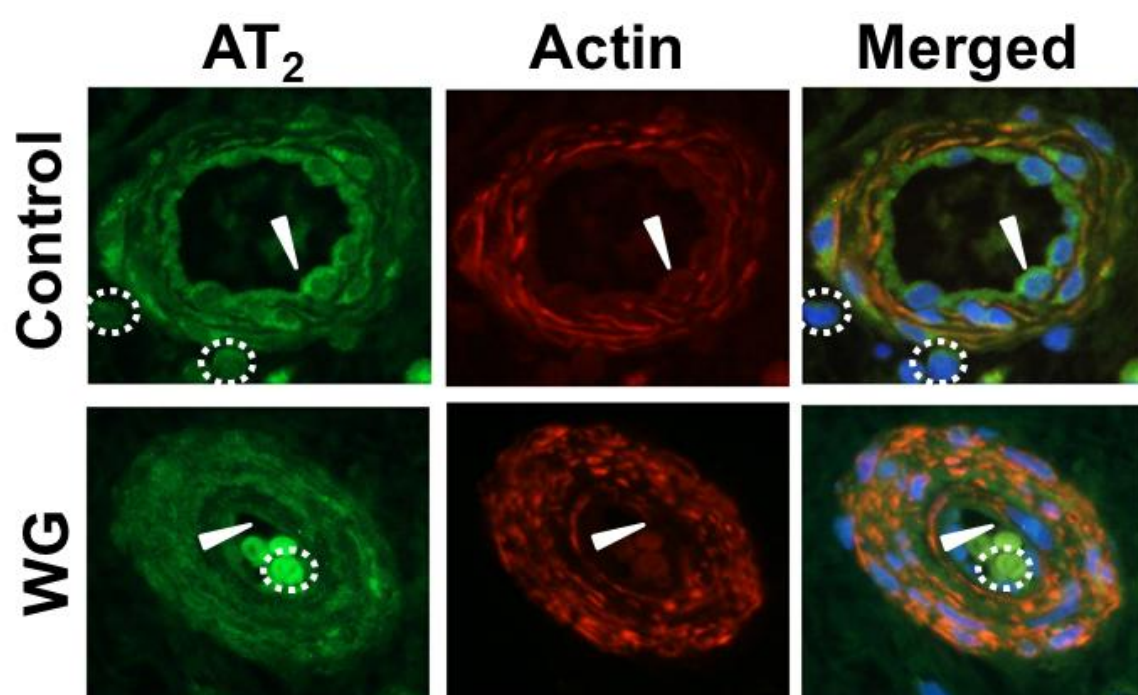
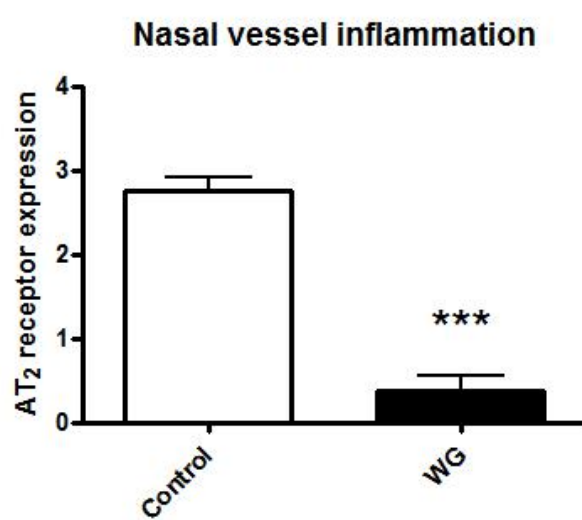
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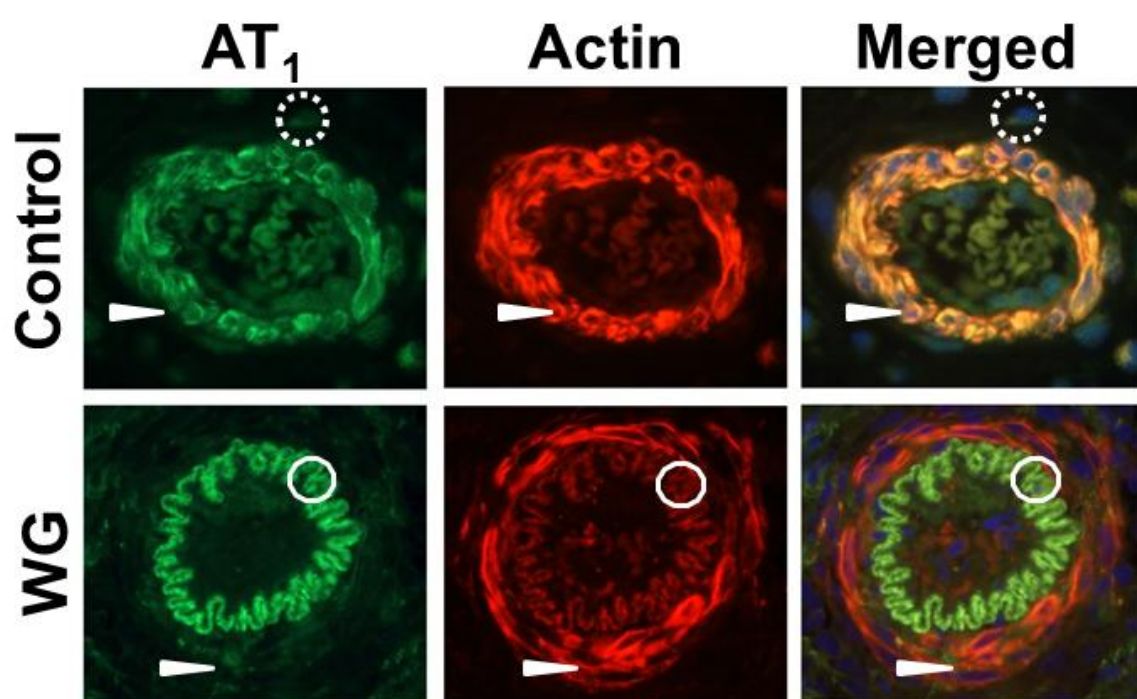
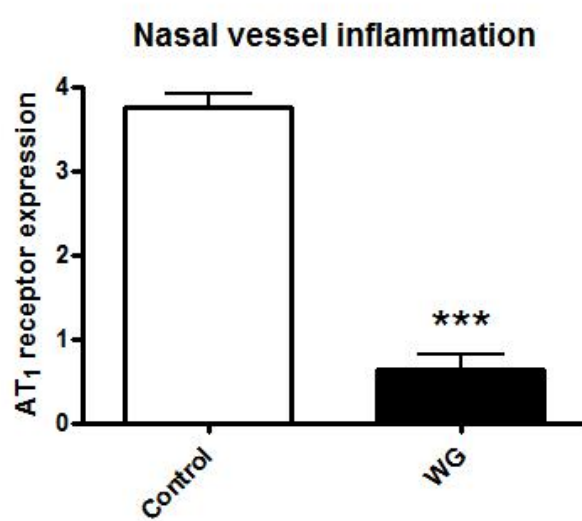
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Figure legends

Figure 1: Immunostaining with antibodies directed against Angiotensin II (ANGII) in arteries from nasal mucus membranes in control patients (control) and patients with granulomatosis with polyangiitis (Wegener's) (GPA) in panel A. The top panel is representative of immunofluorescent images of control vessels (n=8). White isosceles triangles show the endothelial cell layer (EC). Note the ANGIO expression in endothelial cells stained in green and the how staining is reduced in the EC layer in patients with GPA (lower panel). Inflammatory cells, both intra luminal and extra luminal express the ANGIO peptide in the cytoplasm (white dotted circle).

Panel B represent the relative immunofluorescent staining score of EC in nasal resistance artery walls in controls and in patients with GPA. Immunostaining score is presented as the mean according to number of positively stained cells: 0=none, 1 =cells in 25% of the EC or less stained, 2= 25-50% of the EC stained, 3=cells in 50 to 75% of the EC stained, 4 = 75 to 100% of the EC stained is indicated in the figure. Data is presented as a score and mean values \pm standard error of the mean (SEM). Statistical analyses were performed using group mean values and compared by non-parametric Mann–Whitney U test. Significance was defined as $p < 0.001$ (***).(Original magnification, x40).

Figure 2: Immunostaining with antibodies directed against AT₁ receptors in arteries from nasal mucus membranes in control patients (control) and patients with granulomatosis with polyangiitis (Wegener's) (GPA), panel A. The top panel is representative of immunofluorescent images of control vessels (n=8). Note the intense expression of AT₁ receptors in vascular smooth muscle cells (VSMC) stained in green (white isosceles triangles) and the how the immunostaining is lost in the VSMC layer in patients with GPA (lower panel). Observe the auto fluorescence in the lamina elastic interna in the GPA patient (white round circle). Inflammatory cells, both intraluminal and extraluminal, express AT₁ receptors in the cytoplasm (white dotted circles). Panel B represent the relative immunofluorescent

staining score of VSMC cells in nasal resistance artery walls in controls and in patients with GPA. Immunostaining score is presented as the mean of the number of positively stained cells for AT₁ receptors: 0=none, 1 =cells in 25% of the VSMC or less stained, 2= 25-50% of the VSMC stained, 3=cells in 50 to 75% of the VSMC stained, 4 = 75 to 100% of the VSMC stained is indicated in the figure. Data is presented as a score and mean values \pm standard error of the mean (SEM). Statistical analyses were performed using group mean values and compared by non-parametric Mann–Whitney U test. Significance was defined as $P < 0.001$ (***). (Original magnification, x40).

Figure 3: Immunostaining with antibodies directed against AT₂ receptors in arteries from nasal mucus membranes in control patients (control) and patients with granulomatosis with polyangiitis (Wegener's) (GPA), panel A. The top panel is representative of immunofluorescent images of control vessels (n=8). Note the immunostaining of the AT₂ receptors in endothelial cells (EC) stained in green (white isosceles triangles) and the reduction of positively immunostained EC in patients with GPA (lower panel). Inflammatory cells express AT₂ receptors (white dotted circles). Panel B represent the relative immunofluorescent staining score of AT₂ receptors in EC in nasal resistance artery walls in controls and in patients with GPA. Immunostaining score is presented as the mean of the number of positively stained cells for AT₂ receptors: 0=none, 1 =cells in 25% of the EC or less stained, 2= 25-50% of the EC stained, 3=cells in 50 to 75% of the EC stained, 4 = 75 to 100% of the EC stained is indicated in the figure. Data is presented as a score and mean values \pm standard error of the mean (SEM). Statistical analyses were performed using group mean values and compared by non-parametric Mann-Whitney U test. Significance was defined as $P < 0.001$ (***). (Original magnification, x40).