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Neutrophil Extracellular Traps in Acute Chorioamnionitis: A New Mechanism of Host Defense

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ABSTRACT

Problem: Neutrophil extracellular traps (NETs) were recently described as a mechanism for microbial killing in the amniotic cavity of women with intra-amniotic infection. Such a clinical condition can result in acute chorioamnionitis, a placental lesion characterized by the infiltration of maternal neutrophils in the chorioamniotic membranes. Herein, we investigated whether these infiltrating neutrophils form NETs in the chorioamniotic membranes from women who underwent spontaneous term or preterm labor with acute chorioamnionitis.

Method of Study: Chorioamniotic membrane samples were collected from women who underwent spontaneous term or preterm labor with acute chorioamnionitis (n=10 each). Controls included chorioamniotic membrane samples from women who delivered at term or preterm with or without labor, in the absence of acute chorioamnionitis (n=10 each). NETs were visualized and semi-quantified in the chorioamniotic membranes by using antibodies against neutrophil elastase and histone H3, in combination with DAPI staining.

Results: NETs were abundant in the chorioamniotic membranes from women who underwent spontaneous term or preterm labor with acute chorioamnionitis. NETs were rarely found, or not

visualized at all, in the chorioamniotic membranes from women who delivered at term or preterm with or without labor, in the absence of acute chorioamnionitis.

Conclusion: NETs are abundant in the chorioamniotic membranes from women who underwent spontaneous term or preterm labor with acute chorioamnionitis. These findings suggest that chorioamniotic neutrophils form NETs as a mechanism of host defense against infection or danger signals. **Keywords:** Amniotic fluid, alarmins, cytokines, DAMPs, DNA, elastase, fetal inflammatory response, fever, funisitis, histone, intra-amniotic inflammation, microbial invasion of the amniotic cavity, parturition, pregnancy, prematurity, preterm birth, sterile intra-amniotic inflammation **INTRODUCTION**

Acute chorioamnionitis is strongly associated with spontaneous preterm labor[1-4]; yet, it is also frequently observed in the placentas of women who delivered after spontaneous labor at term[5, 6]. In both spontaneous preterm and term labor, this placental lesion is associated with elevated concentrations of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-8, and IL-6 in the amniotic fluid[7-26] and umbilical cord blood[27-33]. Elevated concentrations of such cytokines are linked to adverse neonatal outcomes[20, 21, 27, 34-45]. Therefore, the study herein focused on the mechanisms implicated in acute chorioamnionitis.

The defining morphologic feature of acute chorioamnionitis is diffuse infiltration of neutrophils into the chorioamniotic membranes[46, 47]. Neutrophils are rarely seen in the chorioamniotic membranes in cases without acute chorioamnionitis[47]; therefore, we refer to these innate immune cells as chorioamniotic neutrophils. Their maternal origin was observed when two X chromosomes were detected by fluorescence *in situ* hybridization in the chorioamniotic leukocytes of women who delivered male preterm neonates and whose placenta was diagnosed with acute chorioamnionitis[48, 49]. The current hypothesis[47] states that such maternal neutrophils migrate from the decidual vessels towards the chorion and amnion following a chemotactic gradient established by amniotic fluid chemokines such as IL-8[12, 13, 19, 21, 22, 50-58], CXCL6[59] and GROα[52, 60]. Since acute chorioamnionitis generally represents the presence of intra-amniotic infection[47, 61, 62], we propose that chorioamniotic neutrophils play a role in the maternal host response against microbes invading the amniotic cavity.

In line with our hypothesis, we recently demonstrated that amniotic fluid neutrophils form neutrophil extracellular traps (NETs) as a mechanism for microbial killing in cases with intra-amniotic infection[63]. NETs were initially described as web-like structures that contain

DNA, histones, and anti-microbial products such as neutrophil elastase[64]. NET formation is a specialized cell death process, which represents the final containment effort of a neutrophil to lyse pathogens[65]. Although NET formation (or NETosis[66]) was initially described as an *in vitro* phenomenon[64], *in vivo* NETosis can occur in tissues[64] and intravascular[67]/extravascular fluids (e.g. amniotic fluid[63]). *In vitro*-induced NETs release their components freely as those traps formed in intravascular/extravascular fluids[68, 69]. However, tissue NETs display a unique appearance in each tissue since the release of their components is restricted by the surrounding cellular structures[68, 69]. Tissue NETs are generated in response to a local infection whereas intravascular NETs are formed in response to a systemic infection (i.e. sepsis)[70]. Since acute chorioamnionitis represents the presence of a local inflammatory response in the amniotic cavity, we investigated whether infiltrating neutrophils form NETs in the chorioamniotic membranes.

MATERIALS AND METHODS

Human subjects, clinical specimens, and definitions

Chorioamniotic membrane samples were obtained from the Bank of Biological Specimens of the Detroit Medical Center, Wayne State University, and the Perinatology Research Branch (Detroit, MI, USA), an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services (NICHD/NIH/DHHS). The collection and utilization of biological materials for research purposes were approved by the Institutional Review Boards of Wayne State University and NICHD. All participating women provided written informed consent. The following six study groups were included: 1) women who delivered at term without labor (TNL, n=10); 2) women who underwent spontaneous labor at term without acute chorioamnionitis (TIL, n=10); 3) women who underwent spontaneous labor at term with acute chorioamnionitis (TIL-ACA, n=10); 4) women who delivered preterm without labor (PTNL, n=10); 5) women who underwent spontaneous preterm labor without acute chorioamnionitis (PTL, n=10); and 6) women who underwent spontaneous preterm labor with acute chorioamnionitis (PTL-ACA, n=10). Table I includes the demographic and clinical characteristics of the study population**.** Multiparous women or women with neonates having congenital or chromosomal abnormalities were excluded. Labor at term was defined by the

presence of regular uterine contractions at a frequency of at least two contractions every 10 minutes with cervical changes resulting in delivery. Preterm labor was diagnosed by the presence of regular uterine contractions (at least 3 in 30 min) and documented cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. Preterm delivery was defined as birth prior to the 37th week of gestation.

Placental histopathological examinations

Five-µm-thick sections of formalin-fixed, paraffin-embedded tissue specimens were cut and mounted on SuperFrost™ Plus microscope slides (Erie Scientific LLC, Portsmouth, NH, USA). In each case, several tissue sections of the chorioamniotic membranes, umbilical cord, and placental disc were examined. After deparaffinization, slides were rehydrated, stained with hematoxylin-eosin, and evaluated by pathologists who had been blinded to the clinical outcome, according to published criteria[47, 71]. Acute chorioamnionitis was diagnosed when the infiltration of neutrophils was observed in the chorionic trophoblast layer or chorioamniotic connective tissue[47, 71].

Identification of neutrophil extracellular traps in the chorioamniotic membranes

Chorioamniotic membrane samples were frozen in Tissue-Plus O.C.T. compound (Fisher HealthCare, Houston, TX, USA) immediately after collection. Cryogenic sections were cut to 8 µm and placed on glass microscope slides (Fisherbrand Superfrost Plus slides; Thermo Scientific, Waltham, MA, USA). The sections were fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 20 min at room temperature and rinsed with 1X phosphate-buffered saline (PBS, Life Technologies, Grand Island, NY, USA). Prior to staining, non-specific antibody interactions were blocked using serum-free Protein Blocker (Cat#X09090; DAKO North America, Carpinteria, CA, USA) for 30 min at room temperature. The slides were then incubated at 4°C overnight with a mouse anti-human neutrophil elastase (Cat# M0752, clone NP57, DAKO, Denmark) and a rabbit anti-histone H3 antibody (Cat# ab5103, Abcam, Cambridge, MA, USA). Mouse IgG and rabbit IgG were used as negative controls, respectively. Following staining, slides were washed with 1X PBS with 0.1% Tween 20. Next, a second blocking step was performed by adding 10% goat serum (KPL, Gaithersburg, MD, USA) for 10 mins at room temperature. The slides were then incubated with a secondary goat anti-mouse IgG–Alexa Fluor 488 antibody (Cat# A11029, Life Technologies) and a goat anti-rabbit IgG–

Alexa Fluor 594 antibody (Cat#A11072, Life Technologies) for 30mins at room temperature in the dark. Finally, slides were washed with 1X PBS and mounted with ProLong Diamond Antifade Mountant with DAPI. Slides were visualized on a Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at the Microscopy, Imaging, and Cytometry Resources Core at the Wayne State University School of Medicine (http://micr.med.wayne.edu/). Tile-scans were performed from the chorioamniotic membranes and the complete imaging field was divided into eight by eight quadrants. Z-stack scans (8μm deep) were performed in order to create 3D reconstructions.

Semi-quantification of neutrophil extracellular traps in the chorioamniotic membranes

Following immunostaining, tissue slides were scanned using a Pannoramic MIDI Digital Slide Scanner (PerkinElmer, Inc., Waltham, MA, USA). The chorioamniotic membrane section was divided into quadrants using the scanner software (3DHISTECH Ltd., Budapest, Hungary), and NET semi-quantification was performed in two opposing quadrants. Within each quadrant, five 1mm-wide sections of chorion and five 1mm-wide sections of amnion were chosen in pairs. The width of each section remained constant while the height spanned the full thickness of the amnion or chorion. A NET was defined as a structure in which blue (DAPI), green (neutrophil elastase), and red (histone H3) fluorescence signals were co-localized. The total number of NETs was semi-quantified in the amnion and choriodecidua.

Statistical analyses

The SPSS v.19.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze demographic, clinical and NET semi-quantification data. Normality of the data was tested using the Wilk-Shapiro test. Comparisons among groups were performed using the Kruskal–Wallis test followed by two group comparisons using the Mann-Whitney U-test. Comparison of proportions was made using the Fisher's exact test. A p-value of <0.05 was used to determine statistical significance.

RESULTS

NETs were abundant in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute chorioamnionitis (TIL-ACA; Figure 1A). However, NETs were rarely seen, or not found at all, in the chorioamniotic membranes from women who

underwent spontaneous labor at term without acute chorioamnionitis or those who delivered at term without labor (TIL and TNL; Figure 1A). Magnifications of the NETs found in the chorioamniotic membranes from women who underwent spontaneous term labor with acute chorioamnionitis demonstrated that these traps contain neutrophil elastase and histone H3 (white arrows; Figure 1B), as well as DNA (white arrows; Figure 1C). Semi-quantification revealed that NETs were more abundant in the amnion (Figure 1D) and choriodecidua (Figure 1E) from women who underwent spontaneous labor at term with acute chorioamnionitis than in those without this placental lesion who delivered at term with or without labor. A 3D reconstruction shows that NETs are located in the amnion, chorion and decidua layers of the chorioamniotic membranes from women who underwent spontaneous labor at term with acute chorioamnionitis (Video 1). A snapshot of this 3D reconstruction shows that chorioamniotic membrane NETs contain neutrophil elastase and histone H3 (white arrows; Figure 2A) as well as DNA (white arrows; Figure 2B).

NETs were also abundant in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute chorioamnionitis (PTL-ACA; Figure 3A). However, NETs were rarely seen, or not found at all, in the chorioamniotic membranes from women who underwent spontaneous preterm labor without acute chorioamnionitis or those who delivered preterm without labor (PTL and PTNL; Figure 3A). Magnifications of the NETs found in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute chorioamnionitis demonstrated that these traps contain neutrophil elastase and histone H3 (white arrows; Figure 3B), as well as DNA (white arrows; Figure 3C). Semi-quantification revealed that NETs were more abundant in the amnion (Figure 3D) and choriodecidua (Figure 3E) from women who underwent spontaneous preterm labor with acute chorioamnionitis than in those without this placental lesion who delivered preterm with or without labor. A 3D reconstruction shows that NETs are located in the amnion and choriodecidua from women who underwent spontaneous preterm labor with acute chorioamnionitis (Video 2). A snapshot of this 3D reconstruction shows that chorioamniotic membrane NETs contain neutrophil elastase and histone H3 (white arrows; Figure 4A) as well as DNA (white arrows; Figure 4B).

DISCUSSION

Acute chorioamnionitis generally represents the presence of intra-amniotic infection[47, 61, 62], a clinical condition that is characterized by a local inflammatory response containing abundant leukocytes[72-76] and elevated concentrations of pro-inflammatory mediators such as cytokines[25, 58]. Recently, we characterized the cellular composition of this local inflammatory response using immunophenotyping[77]. We found that neutrophils are the most abundant leukocyte subset in the amniotic cavity of women with intra-amniotic infection[77], which is consistent with previous observations[72]. Such neutrophils mainly express pro-inflammatory cytokines such as TNF-α, MIP-1β, and IL-8[77]. These cytokines are implicated in the processes of term and preterm parturition[10-13, 25, 78-82]. In addition, amniotic fluid neutrophils form NETs in cases with intra-amniotic infection, which represents a new mechanism for trapping and/or killing microbes invading the amniotic cavity[63]. Amniotic fluid neutrophils are considered to be of fetal origin[83, 84]; however, these innate immune cells have also been observed in cases with a severe maternal inflammatory response (i.e. acute chorioamnionitis) without a fetal inflammatory response (i.e. funisitis and chorionic vasculitis), suggesting that, in some cases, amniotic fluid neutrophils are of maternal origin or a mixture of both fetal and maternal neutrophils. In such cases, maternal neutrophils could be migrating from the decidual vessels into the chorion and amnion, causing acute inflammation of the chorioamniotic membranes (i.e. acute chorioamnionitis)[47] and ultimately reaching the amniotic cavity. Therefore, the function of chorioamniotic neutrophils in acute chorioamnionitis may be comparable to their role in intra-amniotic infection since, in both pathological processes, these innate immune cells form NETs and may participate in the maternal host response against microbes invading the amniotic cavity.

Yet, acute chorioamnionitis can also occur in the setting of sterile intra-amniotic inflammation[61, 85-88], an inflammatory process in which microorganisms cannot be detected using a combination of cultivation and molecular microbiology techniques[61, 85-87]. Sterile inflammation is induced by danger signals termed damage-associated molecular patterns (DAMPs)[89] or alarmins[90], derived from necrotic cells or cellular stress[91]. NETs can also be formed in sterile inflammation since both alarmins and pathogen-associated molecular patterns (PAMPs) use the same sensor molecules or pattern recognition receptors[92]. Particularly, high-mobility group box-1 (HMGB1, a prototypical alarmin[93, 94]) can induce NET formation via TLR4[95], which is the sensor molecule for lipopolysaccharide from Gram negative bacteria[96]. The fact that HMGB1 induces NETs is relevant since: (1) amniotic fluid HMGB1 concentrations are higher in women

with intra-amniotic infection[97] or clinical chorioamnionitis[98] than in those without these clinical conditions; (2) patients with sterile intra-amniotic inflammation and high amniotic fluid HMGB1 concentrations delivered earlier than those with low concentrations of this alarmin[85]; (3) the intra-amniotic administration of HMGB1 induces preterm labor and birth in mice[99]; and (4) the chorioamniotic membranes from women who underwent spontaneous preterm labor releases high concentrations of HMGB1[100]. Alarmin-induced NETs can exacerbate immune responses by directly causing tissue damage[92]. Together, these data suggest that in the setting of sterile intra-amniotic inflammation, chorioamniotic neutrophils form NETs in response to danger signals derived from the amniotic fluid or the chorioamniotic membranes which, in turn, could aggravate the local immune response observed in acute chorioamnionitis.

In summary, the study herein demonstrated that neutrophils infiltrating the chorioamniotic membranes in preterm and term cases of acute chorioamnionitis form NETs. These data suggest that chorioamniotic neutrophils form NETs in response to microbes invading the amniotic cavity (i.e. intra-amniotic infection) or danger signals derived from the amniotic fluid or chorioamniotic membranes (i.e. sterile intra-amniotic inflammation). Collectively, these findings provide insight into the functions of infiltrating neutrophils in the chorioamniotic membranes from women who underwent spontaneous term or preterm labor with acute chorioamnionitis.

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Disclosure/Conflict of Interest

The authors disclose no conflicts of interest.

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FIGURE LEGENDS

Figure 1. NETs in the chorioamniotic membranes from women who delivered at term. (A) A tile-scan image of the chorioamniotic membranes from women who delivered at term without labor (TNL) or underwent spontaneous labor at term with (TIL-ACA) or without (TIL) acute chorioamnionitis. Merged images show DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red. Tile-scan images were acquired at 400X. The area outlined in (A) is enlarged in (B&C), demonstrating a higher resolution view of the NETs in the choriodecidua from women who underwent spontaneous labor at term with acute chorioamnionitis (TIL-ACA). Merged images show neutrophil elastase in green and histone H3 in red (B; white arrows) or DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red (C; white arrows).

NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized. Semi-quantification of the total number of NETs in the amnion (D) and choriodecidua (E).

Video 1. 3D reconstruction of NETs in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute chorioamnionitis. Merged image shows DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red. 400X magnifications. NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized.

Figure 2. A snapshot of the 3D reconstruction of NETs in the chorioamniotic membranes from women who underwent spontaneous labor at term with acute chorioamnionitis (TLA-ACA). Merged image shows neutrophil elastase in green and histone H3 in red (A; white arrows). Merged image shows DAPI (nuclei) in blue, neutrophil elastase in green and histone H3 in red (B; white arrows). 400 X magnifications. NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized.

Figure 3. NETs in the chorioamniotic membranes from women who delivered preterm. (A) A tile-scan image of the chorioamniotic membranes from women who delivered at term without labor (PTNL) or underwent spontaneous labor at term with (PTL-ACA) or without (PTL) acute chorioamnionitis. Merged images show DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red. Tile-scan images were acquired at 400X. The area outlined in (A) is enlarged in (B&C), demonstrating a higher resolution view of the NETs in the choriodecidua from women who underwent spontaneous labor at term with acute chorioamnionitis (TIL-ACA). Merged images show neutrophil elastase in green and histone H3 in red (B; white arrows) or DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red (C; white arrows). NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized. Semi-quantification of the total number of NETs in the amnion (D) and choriodecidua (E).

Video 2. 3D reconstruction of NETs in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute chorioamnionitis. Merged image shows DAPI (nuclei) in blue, neutrophil elastase in green, and histone H3 in red. 400X magnifications. NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized.

Figure 4. A snapshot of the 3D reconstruction of NETs in the chorioamniotic membranes from women who underwent spontaneous preterm labor with acute chorioamnionitis (PTL-ACA). Merged image shows neutrophil elastase in green and histone H3 in red (A; white arrows). Merged image shows DAPI (nuclei) in blue, neutrophil elastase in green and histone H3 in red (B; white arrows). 400 X magnifications. NETs are structures in which DAPI, neutrophil elastase, and histone H3 fluorescence signals are co-localized.

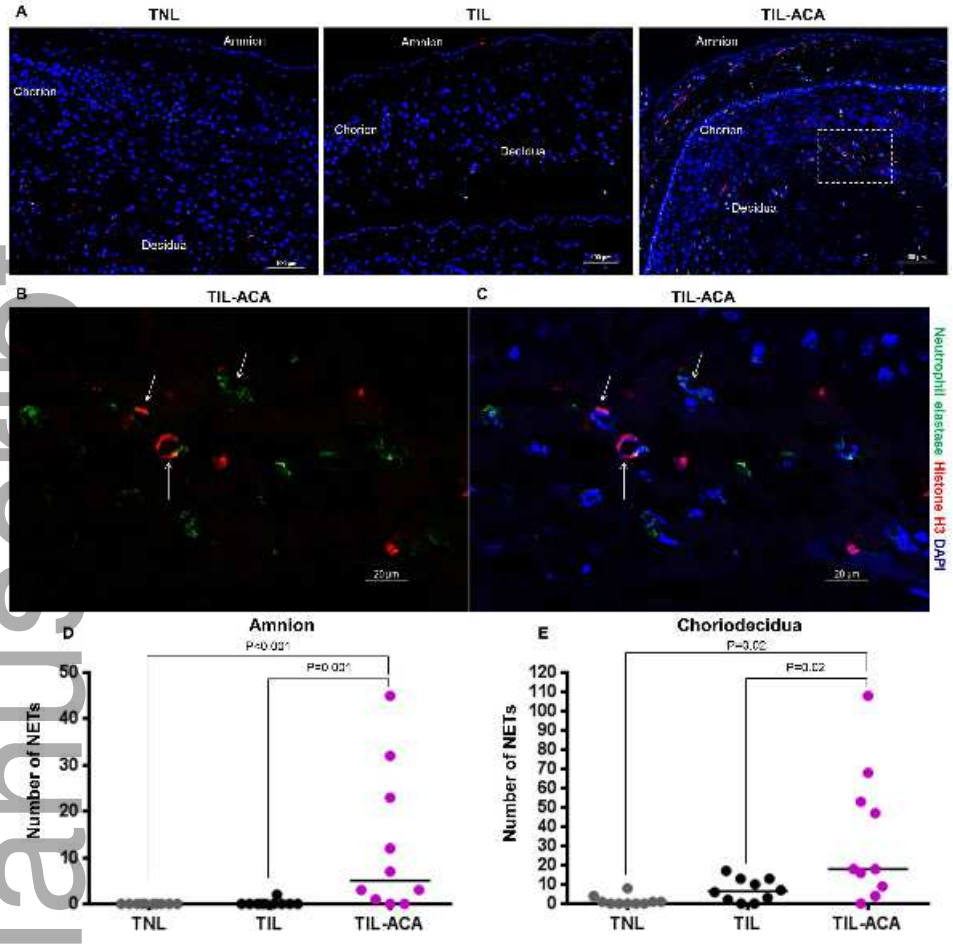
Table I. Demographic and clinical characteristics of the study population

	TNL (n=10)	TIL (n=10)	TIL-ACA (n=10)	PTNL (n=10)	PTL (n=10)	PTL-ACA (n=10)	p value
Age (y; median [IQR])[a]	28 (25.3-29.8)	24.5 (21.5-31.3)	22.5 (20.5-26)	29 (22.5-33)	25.5 (22.5-28.3)	27 (21.5-30.8)	NS
Body mass index (kg/m^2; median [IQR])[a]	33.7 (29.8-36.6)	23 (21.1-27.2)	27.9 (24.6-29)	27.5 (21.9-32.8)	23.4 (22.7-27.3)	25.2 (21.4-27.1)	p=0.037
Gestational age at delivery (wk; median [IQR])[a]	39.3 (39-39.6)	39.2 (38.9-39.9)	39.2 (38.8-40)	31 (29.7-32.6)	33.6 (32.8-34.1)	32.75 (31.6-34.5)	p<0.001
Birth weight (g; median [IQR])[a]	3692.5 (3006.3-3853.8)	3145 (2677.5-3255.75)	3322.5 (3093.8-3476.3)	1170 (931.3-1290)	1895 (1717.5-2078.8)	1827.5 (1517.5-2848.8)	p<0.001
Race (n[%])[b]							
African-American	6 (60%)	10 (100%)	9 (90%)	8 (80%)	10 (100%)	7 (70%)	NS
Caucasian	3 (30%)	0 (0%)	1 (10%)	1 (10%)	0 (0%)	1 (10%)	
Other	1 (10%)	0 (0%)	0 (0%)	1 (10%)	0 (0%)	2 (20%)	
Primiparity (n[%])[b]	0 (0%)	3 (30%)	1 (10%)	1 (10%)	1 (10%)	1 (10%)	NS
C-section (n[%])[b]	10 (100%)	2 (20%)	1 (10%)	10 (100%)	4 (40%)	1 (10%)	NS
Acute fetal inflammatory response (n[%])[b]							
Stage 1 (acute phlebitis/chorionic vasculitis)	0 (0%)	0 (0%)	2 (20%)	0 (0%)	0 (0%)	4 (40%)	NS
Stage 2 (acute arteritis)	0 (0%)	0 (0%)	7 (70%)	0 (0%)	0 (0%)	3 (30%)	NS
Stage 3 (necrotizing funisitis)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)	NS

[a]Kruskal-Wallis test

[b]Fisher's exact test

IQR = interquartile range



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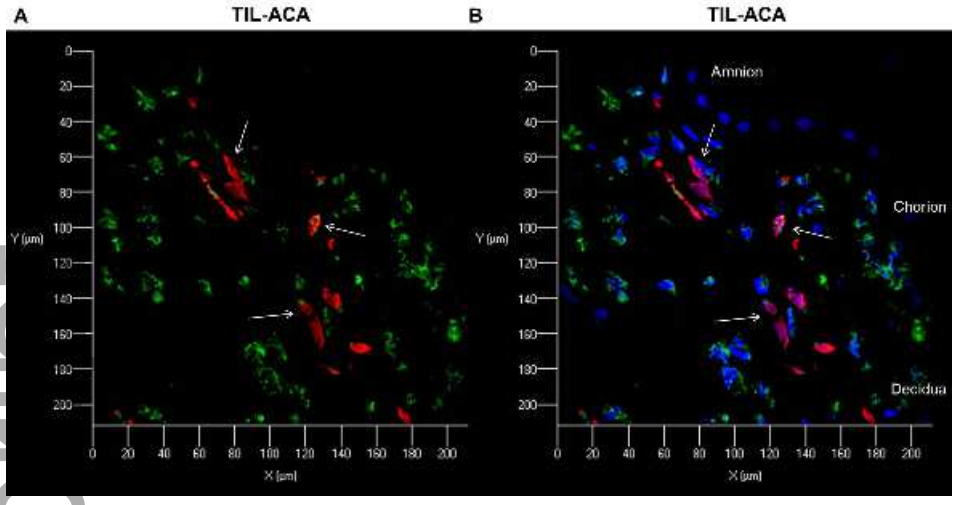

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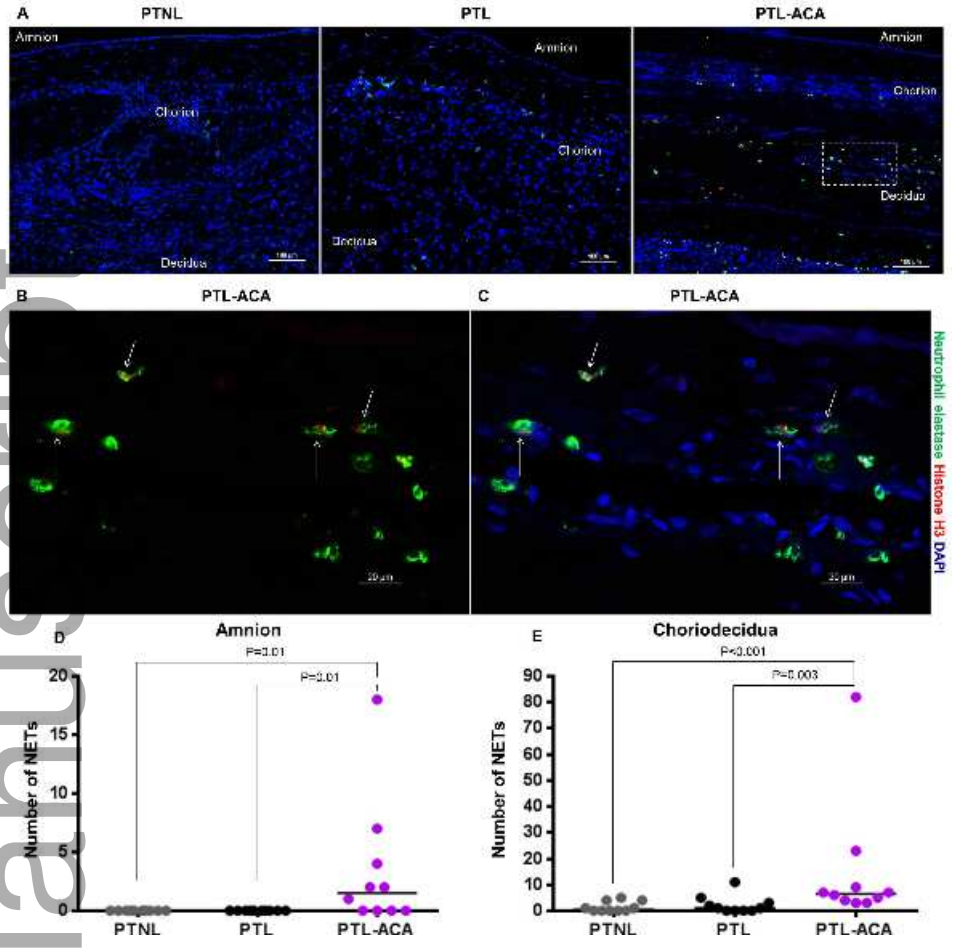

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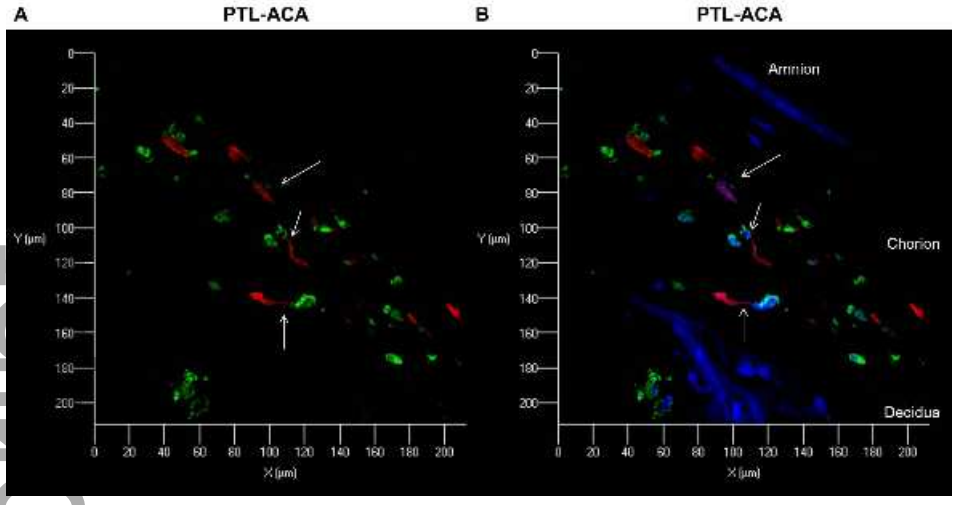

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