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Technical note

Both "canonical" and "immunological" preeclampsia subtypes demonstrate changes in placental immune cell composition

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ABSTRACT

Our prior work investigating the heterogeneity of preeclampsia identified multiple placental subtypes of this disorder, including a "canonical" group with maternal vascular malperfusion and an "immunological" group with signs of allograft rejection. Here, we perform a pilot immunohistochemistry study to investigate if an increase in infiltrating maternal immune cells is contributing to the "immunological" pathology subtype. This revealed an enrichment of monocytes and/or neutrophils (CD68$^+$ and MPO$^+$ cells) in the intervillous space of these placentas. Surprisingly, "canonical" samples also demonstrated a significant result, with decreased CD68 staining. As such, further immunohistochemistry to assess immune contributions in preeclampsia subtypes is warranted.

1. Introduction

Preeclampsia (PE) is a complex and heterogeneous hypertensive disorder of pregnancy. Previously, we addressed this heterogeneity by subjecting placental gene expression data from PE and non-PE pregnancies to unsupervised clustering (an unbiased method of grouping samples) [1–4]. This analysis revealed three clinically, epigenetically, and histologically relevant subtypes of PE placentas, belonging to transcriptional clusters 1, 2, and 3 [4–6]. Within cluster 1, the PE samples were relatively healthy, with transcriptional similarity to term controls and minimal histopathology. Cluster 2 placentas revealed substantial "canonical" features of maternal vascular malperfusion, such as accelerated villous maturity. Finally, cluster 3 samples displayed signs of an "immunological" pathology, involving a transcriptional and epigenetic signature of heightened immune response and histological lesions affiliated with chronic inflammation and allograft rejection. However, surprisingly, a large portion of the genes and DNA methylation marks found to be enriched in this "immunological" cluster are not normally present in placental tissue and, instead, are expected to be expressed by immune cells [3–5,7]. Here, we conduct a pilot immunohistochemistry study to test the hypothesis that an increased number of infiltrating maternal immune cells are significantly contributing to the immune response signature observed in the cluster 3 placentas.

2. Methods

2.1. Sample selection and immunohistochemistry

A total of 15 placental samples (five from transcriptional cluster 1, five from transcriptional cluster 2, and five from transcriptional cluster 3), covering a representative range of clinical outcome groups, were selected from our previously published preeclampsia-focused cohort (Supplementary Table 1) [4]. The characteristics of this cohort, as well as the placental sampling methods and gene expression findings, have been previously described [4,6,8]. For this study, seven sections of each placental biopsy (four biopsies per placenta) were cut onto slides and stained with an antibody for cluster of differentiation (CD) 3 (T cells), CD4 (CD4$^+$ ("helper") T-cells), CD8 (CD8$^+$ ("cytotoxic") T-cells), CD20 (B cells), CD56 (natural killer cells), CD68 (monocytes/macrophages), or myeloperoxidase (MPO; neutrophils) [9–11]. Further details are available in the Supplementary Methods and positive controls are shown in Supplementary Fig. 1.

Abbreviations: CD, cluster of differentiation; IVS, intervillous space; MPO, myeloperoxidase; PE, preeclampsia

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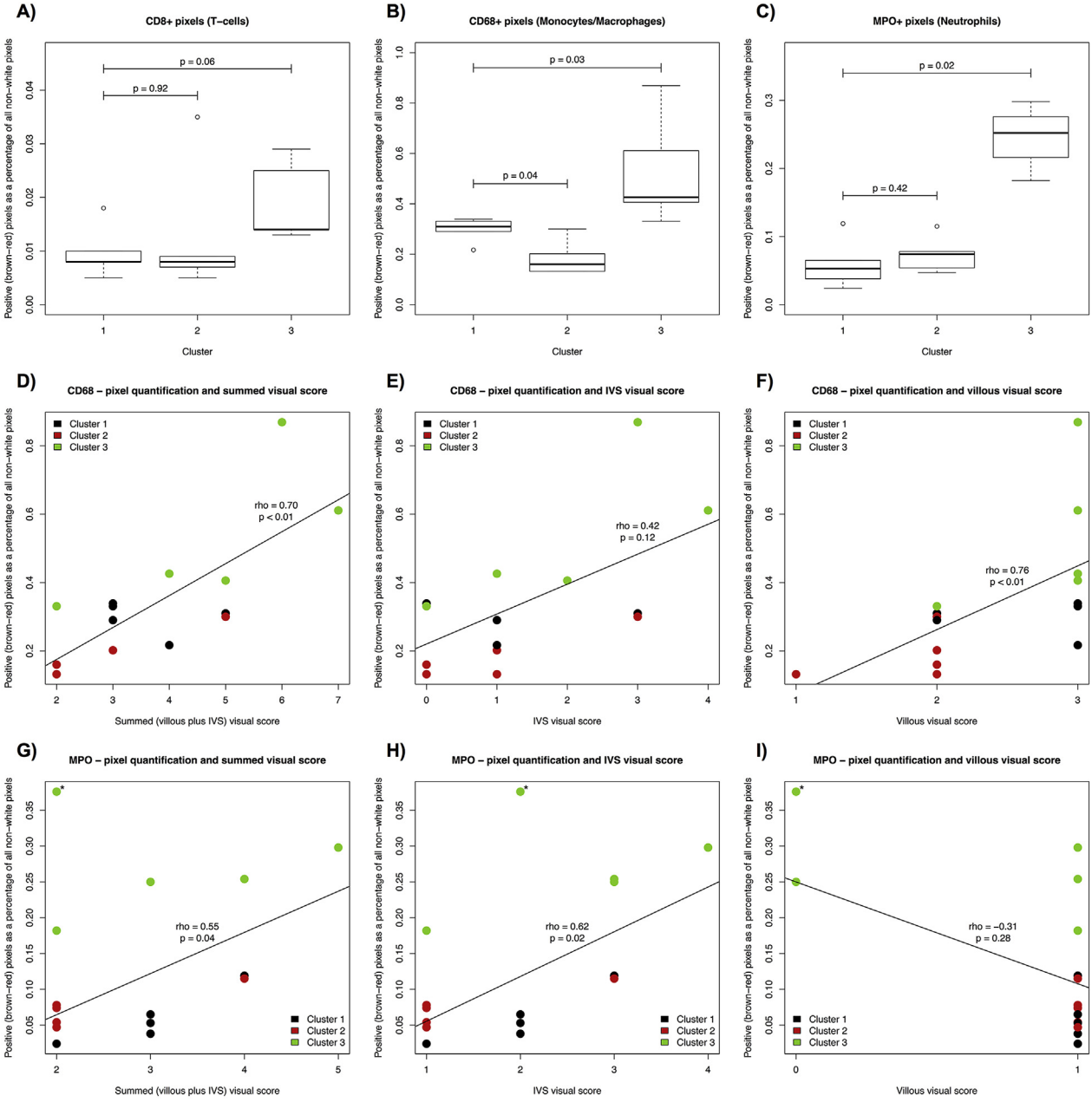

Fig. 1. Significant pixel quantification results and correlations with visual scores. Compared to transcriptional cluster 1 placentas, cluster 3 samples demonstrated increased (**A**) $CD8^+$ T-cells ($p = 0.06$), (**B**) $CD68^+$ monocytes/macrophages ($p = 0.03$), and (**C**) myeloperoxidase (MPO)+ neutrophils ($p = 0.02$) by pixel quantification. Cluster 2 placentas revealed a decrease in CD68 staining (**B**) ($p = 0.04$). (**D**) The pixel quantification and the summed (intervillous space (IVS) plus villous space) visual scores correlated for CD68 ($p < 0.01$), and this appears to be driven by changes in both (**E**) the IVS ($p = 0.12$) and (**F**) the villous space ($p < 0.01$). (**G**) In contrast, the overall correlation observed between the pixel quantification and summed visual scores for MPO ($p = 0.04$) appears to be driven almost entirely by changes in (**H**) the IVS ($p = 0.02$), with little to no contribution from (**I**) the villous space ($p = 0.28$). In general, cluster 3 placentas tended to be associated with higher pixel quantification values than cluster 1 and 2 samples with the same visual score, likely due to differences in villous density (ex. $p = 0.01$ by Kruskal-Wallis test for the number of non-white pixels across the three clusters in the CD68 sections), confirming the importance of correcting for this in the pixel quantification assessment. In (**G**), (**H**), and (**I**), the asterisk indicates a cluster 3 sample that was excluded from both the pixel quantification analysis (**C**) and the correlation analysis due to high non-specific staining of fibrin in the IVS (Supplementary Fig. 3).

2.2. Image quantification

For each individual stained placental biopsy, pixel counts were obtained for the total image, the white pixels, and the brown-red pixels (indicating a positive stain) in Adobe Photoshop. The number of positively stained pixels was converted to a percentage of all non-white pixels, similar to previously published methods [12], and this value was averaged across the four biopsies associated with each placenta for CD20, CD3, CD8, CD68, and MPO. Quantification was not successful for CD56 or CD4 due to significant background staining. Additional visual scoring, on a scale of 0–5, was also performed by an experienced perinatal pathologist (DG) for the CD68 and MPO images only (staining was too low for CD20, CD3, and CD8). Positive staining was scored separately for the intervillous space (IVS) and the villous space. Further image quantification details are available in the Supplementary Methods.

2.3. Statistical analysis

Differences in immune cells across the transcriptional clusters were determined using Wilcoxon rank-sum tests. Relationships between the pixel quantification and visual scoring results, and the pixel quantification and previously discovered cytokine gene expression values [4], were investigated using Spearman correlations.

3. Results and discussion

Pixel quantification revealed that $CD20^+$ B-cells were not differentially present in transcriptional cluster 2 or 3 placentas compared to the healthier cluster 1 samples ($p = 0.40$ and $p = 0.92$, respectively; Supplementary Fig. 2a). $CD3^+$ T-cells showed a trend towards a higher frequency in clusters 2 and 3, although this was not statistically significant ($p = 0.20$ and $p = 0.29$, respectively, versus cluster 1; Supplementary Fig. 2b). In contrast, $CD8^+$ T-cells, $CD68^+$ monocytes/macrophages, and MPO^+ neutrophils were all increased in cluster 3 placentas, compared to cluster 1 ($p = 0.06$, $p = 0.03$, and $p = 0.02$, respectively; Fig. 1a–c). However, a very different pattern was observed in cluster 2 samples, with similar CD8 ($p = 0.92$) and MPO ($p = 0.42$) staining, but decreased CD68 staining ($p = 0.04$), when assessed against cluster 1 (Fig. 1a–c).

To confirm the pixel quantification findings and identify the placental compartments (IVS and/or villous) responsible for these results, the CD68 and MPO images also underwent a visual assessment (Fig. 2). Overall, the pixel quantification and the summed (IVS plus villous) visual scores correlated for both CD68 (rho = 0.70, $p < 0.01$; Fig. 1d) and MPO (rho = 0.55, $p = 0.04$; Fig. 1g). When the visual scores were broken down into the IVS and villous compartments, CD68 mostly maintained this correlation to the pixel values in both instances (rho = 0.42, $p = 0.12$ and rho = 0.76, $p < 0.01$, respectively; Fig. 1e and f), indicating that changes in both the number of infiltrating maternal monocytes into the IVS and the quantity of fetal villous macrophages (Hofbauer cells) may contribute to the observed decrease in CD68 staining in cluster 2 and increase in CD68 staining in cluster 3. Contrarily, the higher MPO staining in cluster 3 samples appeared to be driven almost entirely by changes in the IVS (rho = 0.62, $p = 0.02$ for the IVS and pixel correlation; rho = −0.31, $p = 0.28$ for the villi and pixel correlation; Fig. 1h and i). This suggests a unique abundance of MPO^+ cells in the IVS of cluster 3 placentas. Furthermore, pixel quantification values for both CD68 and MPO correlated with the expression of a number of different cytokines in these placentas (Supplementary Table 2), affirming that these immune cells are significantly impacting the placental transcriptional signature.

In conclusion, this pilot analysis confirms that an increased number of maternal immune cells are present in the IVS of transcriptional cluster 3 placentas, predominately monocytes and/or neutrophils. As these cell types have known roles in innate immune response, autoimmunity, and preeclampsia [13–16], these findings fit with prior evidence suggesting that cluster 3 placentas are experiencing a maternal allogeneic-type immunological rejection [4,6,17]. Additionally, although the two cluster 3 placentas with the highest CD68 staining had both been previously diagnosed with an inflammatory histopathological lesion (one with villitis of unknown etiology and the other with chronic intervillositis) [6], the remaining three samples fell shy of current diagnostic nosology, indicating that even mild increases in cellular infiltrates can have an important effect on placental gene expression. The decreased CD68 staining in transcriptional cluster 2 samples was somewhat more surprising, but supports the notion that sufficient fetal macrophages/Hofbauer cells are essential for placental health [18]. Furthermore, cluster 2 is characterized by accelerated villous maturation, which does entail increased stromal collagenization, whereas macrophages are most present in immature stroma “stellate reticulum” [19,20]. Overall, however, since different immune cell types can express overlapping markers/CD antigens [9], we cannot be confident that monocytes/macrophages and neutrophils are exclusively driving the current results, or that monocytes aren't responsible for both the enriched CD68 and MPO signal observed [21], which is the primary limitation of this study. Additional validation of these findings, as well as further investigation into the role of immune cells in these two distinct pathological preeclampsia subtypes, is warranted.

Conflicts of interest

The authors report no conflict of interest.

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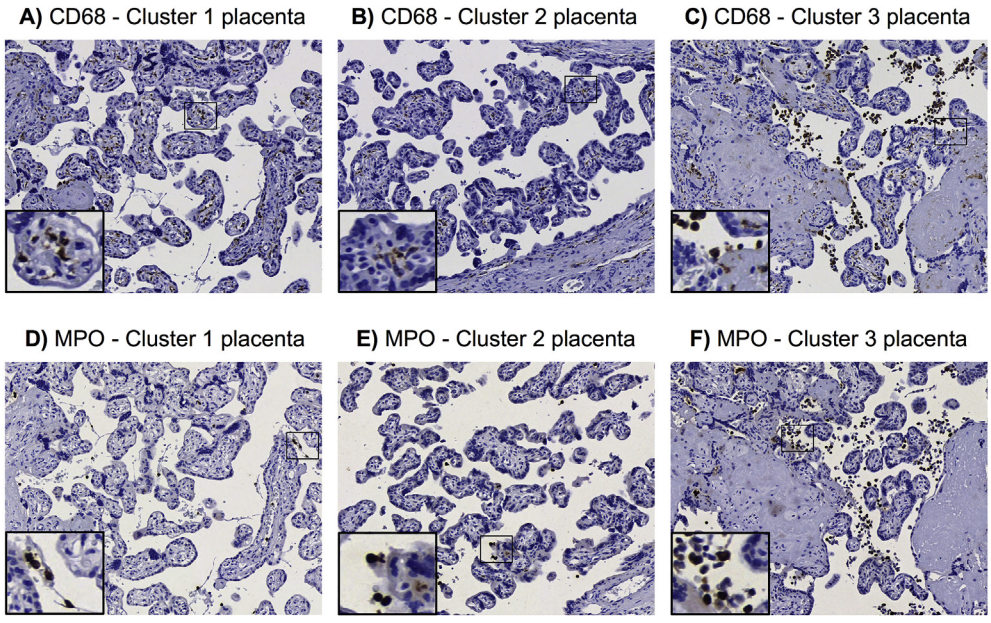

Fig. 2. Representative images of CD68 and MPO staining across the clusters. CD68 staining in (**A**) a cluster 1 placenta, (**B**) a cluster 2 placenta, and (**C**) a cluster 3 placenta. Myeloperoxidase (MPO) staining in the same (**D**) cluster 1 placenta, (**E**) cluster 2 placenta, and (**F**) cluster 3 placenta. Immunohistochemistry was performed using a DAB color reaction (indicated by a brown-red stain) and a hematoxylin counterstain. Images were obtained at 20× magnification. A further magnified portion of the image is shown in the bottom left corner (~60×).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.06.384.

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