*Original Article*

# Mapping of the Human Amniotic Membrane: *In Situ* Detection of Microvesicles Secreted by Amniotic Epithelial Cells

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## Abstract

The potential clinical applications of human amniotic membrane (hAM) and human amniotic epithelial cells (hAECs) in the field of regenerative medicine have been known in literature since long. However, it has yet to be elucidated whether hAM contains different anatomical regions with different plasticity and differentiation potential. Recently, for the first time, we highlighted many differences in terms of morphology, marker expression, and differentiation capabilities among four distinct anatomical regions of hAM, demonstrating peculiar functional features in hAEC populations. The aim of this study was to investigate *in situ* the ultrastructure of the four different regions of hAM by means of transmission electron microscopy (TEM) to deeply understand their peculiar characteristics and to investigate the presence and localization of secretory products because to our knowledge, there are no similar studies in the literature. The results of this study confirm our previous observations of hAM heterogeneity and highlight for the first time that hAM can produce extracellular vesicles (EVs) in a heterogeneous manner. These findings should be considered to increase efficiency of hAM applications within a therapeutic context.

## Keywords

stem cells, amniotic membrane, amniotic epithelial cells, electron microscopy, microvesicles

## Introduction

The human placenta is a transient organ made up of both fetal and maternal components which originate from the blastocyst and the maternal endometrium, respectively[1]. The fetal components include the umbilical cord, the placental disc, the amniotic and chorionic membranes, and the chorionic villi[2,3]. It is well known that human amniotic membrane (hAM) has proven to be a precious resource in the field of regenerative medicine with a long history of therapeutic

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applications[4–8] because it is a biocompatible readily available and cost-effective biological tissue that does not raise any ethical issues[9,10]. Human AM possesses unique biological properties exerting anti-inflammatory, low immunogenicity, antifibrotic, antimicrobial, and antimutagenic effects[8,11–13] and it is a source of growth factors, cytokines, and cells with stemness properties[2,3]. Thanks to advances in stem cell–based approaches, human amniotic epithelial cells (hAECs) have been widely studied for the treatment of various diseases[14], such as lung and liver injury and fibrosis, diabetes, acute kidney failure, cardiovascular diseases, wound healing, and premature ovarian failure (POF)[15–21]. Despite the great therapeutic potential of these cells, preservation and transplantation difficulties make the effective use of hAECs problematic[22,23]. In the last 10 years, increasing number of findings demonstrated that stem cell therapeutic effects are mainly due to a paracrine mechanism where extracellular vesicles (EVs) are involved in tissue/organ regeneration[24]. EVs can be divided into microvesicles (MVs), apoptotic bodies, and exosomes, secreted by nearly all the cells and proved to play a central role in intercellular communication by delivering microRNAs (miRNAs), immunomodulatory proteins, and bioactive lipids from donor cells to recipient cells[25]. Several studies demonstrated that MVs and placenta-derived exosomes are constitutively secreted throughout the pregnancy[26]. It has been demonstrated that placenta-derived exosomes act as initiators of syncytiotrophoblast formation and take part in the fetal allograft survival[27]. Moreover, it has been reported that plasmatic exosomal concentration is elevated in pregnant women delivering at term in comparison with preterm delivery[28]. Interestingly, MVs and placenta-derived exosomes have been also proposed as markers in case of pregnancy complications[29–32]. However, the secretion modalities, the localization, and the role of MVs and placenta-derived exosomes have yet to be determined. Moreover, as reported in the literature, exosome studies are usually carried out using preparations isolated from biological fluids and cell secretome by means of a wide range of methods, including centrifugation followed by ultracentrifugation as well as ultracentrifugation in a density gradient[33]. It should be noted that these methods allow the obtainment of preparations enriched in exosomes, but not homogeneous pure exosome preparations, thus leading to high data variability in exosome molecular composition and lack of important information in deep understanding of their secretion modalities[34]. Furthermore, the commonly used MV isolation methods do not give any information about MV localization, are costly and time-consuming, and require huge numbers of cells to recover enough material for further characterization studies[35]. At present, it is known that both hAECs and mesenchymal stromal cells from the amniotic membrane (hAMSCs) can produce MVs[36], but, to our knowledge, nobody has demonstrated whether MV secretion occurs along the entire AM or rather is restricted to specific regions of AM. In this regard, our research group has recently performed a detailed *in situ* morphofunctional characterization ("mapping") of hAM demonstrating the presence of four different regions (central, intermediate, peripheral, and reflected) (Supplemental Figure S1), according to their position relative to the umbilical cord, displaying hAECs with different morphology, expression of pluripotency markers, and differentiation capabilities[37,38]. Based on these observations, the aim of this study was to investigate *in situ* the ultrastructure of the four different regions of hAM by means of transmission electron microscopy (TEM) to deeply understand their peculiar characteristics and to investigate the presence and localization of secretory products because to our knowledge, there are no similar studies in the literature.

## Materials and Methods

### Subjects and Sample Preparation

Human term placentas (n = 10) were collected from healthy women (mean age ± SD; 33.6 ± 4.8) undergoing cesarean section after obtaining informed written consent according to the guidelines set by the Ethics Committee of the G. d'Annunzio University of Chieti-Pescara, Italy, and the local ethical committee "Comitato Etico Provinciale di Brescia," Italy (protocol number: 191/6.2.17 Tit. III Cl. 13; number NP 2243, January 19, 2016).

Human AM samples were obtained from four different concentric regions of the AM, identified according to their macroscopic characteristics and position relative to the umbilical cord (R1: surrounding the umbilical cord; R2: intermediate between R1 and R3; R3: peripheral to the placental disc; and R4: reflected AM corresponding to chorion laeve) as previously described[37]. A minimum of five samples were prepared for TEM procedures, whereas five samples were submitted to enzymatic digestion to isolate hAECs from four different regions of hAM.

### Isolation of hAECs

Human AM was manually separated from the chorion, extensively washed, and treated with antibiotic and antifungal solutions. Human AECs from different regions were prepared as described by Magatti et al.[39] Briefly, fragments from four different areas of hAM were separately incubated in 1× phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA) containing 0.5-mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) and 100-U/ml penicillin plus 100-mg/ml streptomycin (P/S, Sigma-Aldrich) for 10 min at 37°C and then in 1× trypsin/EDTA solution (Sigma-Aldrich; 10 ml for each fragment) for 5 min at 37°C. After discarding debris, the fragments were incubated once more in fresh trypsin/EDTA solution for 10 min at 37°C. Then, the tissue was carefully shaken, and the trypsin was inactivated by adding three to four volumes of RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 2-mM L-glutamine

(Sigma-Aldrich), and P/S. The fragments were then washed twice in PBS and digested a third time in trypsin/EDTA. The cells from the second and third digests were pooled and centrifuged at 300 × *g* for 10 min. Cell suspensions were then filtered through a 70-mm cell strainer (BD Biosciences, San Jose, CA, USA), centrifuged, counted, and the cell pellets processed for TEM.

### *TEM Procedures*

Human AM samples and hAEC cellular pellets were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1-M cacodylate buffer (Electron Microscopy Sciences) (pH: 7.2–7.4) for 2–3 h at 4°C and postfixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1–2 h at 4°C. After dehydration in progressively higher concentrations of alcohol, samples were embedded in Spurr resin (Electron Microscopy Sciences). Semithin sections (0.7–1 μM) were stained with 1% toluidine blue (Electron Microscopy Sciences) and analyzed with a ZEISS Axioskop light microscope (Carl Zeiss, Gottingen, Germany) equipped with a Coolsnap digital camera (Photometrics, Tucson, AZ, USA). Ultrathin sections (70 nm) were cut with a Reichert ultramicrotome (Reichert, Inc, Teramo, Italy), mounted on 200 mesh copper grids (Electron Microscopy Sciences), and counterstained with UranyLess and lead citrate staining solutions (Electron Microscopy Sciences) for 10–15 min/each. Samples were observed under a ZEISS EM109 electron microscope equipped with a Gatan 830Z00W44 camera (Gatan GmbH, Ingolstadterstr. 12 D-80807 München, Germany) and Digital Micrograph application used for acquiring, visualizing, analyzing, and processing digital image data.

### *Morphometric Analysis*

MetaMorph 6.1 Software System (Universal Imaging Corp, Molecular Device Corp, CA, USA) was employed to acquire digital images and perform morphometric analysis, as already described[40]. Morphometric computerized analysis of about 100 MV mean diameters and the specific vesicle areas and diameters (10 photographic fields at 7,000×; 12,000×; 20,000×; and 50,000× on five sections) were performed manually drawing the area regions and the diameter segments, after calibrating the MetaMorph 6.1r6 program (Universal Imaging Corp) for the magnification used (7,000×; 12,000×; 20,000×; and 50,000×).

## Results

### *Ultrastructural Analysis of Different Regions of hAM*

The ultrastructural study of hAM was performed to investigate the heterogeneity of the amniotic epithelium and to detect detailed aspects of morphological and functional differences among the four different regions of hAM. First, we observed a heterogeneous morphology of hAECs. In fact, in the central area, hAECs possess a regular cell morphology and exhibit mostly a cuboidal shape with a round central nucleus with evident nucleoli as shown in Fig. 1A.

In the apical region, short and blunt microvilli protruding into the amniotic fluid can be observed (Fig. 1B). Interestingly, numerous gaps and lateral microvilli in the intercellular space between the cells are present and some desmosomes can also be observed (arrows) (Fig. 1C). The basal side of the cells is quite irregular due to the presence of numerous cytoplasmic extensions that allow hAECs to enter the basal membrane through which they get in touch with the underlying connective tissue (Fig. 1D).

Several morphological differences can be observed in the intermediate area (Fig. 2). First, hAECs have a dome shape and are smaller compared with the cells in the central area. The microvilli in the apical surface appear thicker, shorter, and less numerous in comparison with those of the central area (Fig. 2B and C), while they are even more numerous in the lateral area. The cytoplasm extensions in the basal lamina are shorter and spaced apart (Fig. 2D).

The epithelial cells in the peripheral area are taller and columnar in shape and their cytoplasm is full of granules of different size, shape, and content (Fig. 3A). The cells appear less close to each other and arranged on more layers compared with hAECs in the central and intermediate areas. Long and thin microvilli both in the apical portion and in the lateral sides are present (Fig. 3B and C). Several mitochondria can also be observed.

Finally, the reflected area shows characteristic features like the central area, such as short microvilli in the apical region (Fig. 4A and B), whereas in the lateral side, microvilli are less numerous in comparison with the central area (Fig. 4C). The presence of intercellular junctions formed by numerous desmosomes can also be appreciated, indicating a strong cellular bond (thin arrows). The cytoplasmic extensions into the basement membrane appear shorter and thicker compared with the other regions of hAM (Fig. 4D).

### *Ultrastructural Analysis of EV Release, Morphology, and Localization*

Thanks to the *in situ* ultrastructural analysis with TEM, we were able to observe some groups of structures with a vesicular aspect (Fig. 5). Interestingly, as shown in Fig. 5, we noticed their presence only in the central region of hAM.

This group of vesicles seemed to derive from the budding at the apical surface of the epithelial cell or from detached parts of the cytoplasm, present in the extracellular space as budding fragments (Fig. 6A and B). Morphometric analysis of the structures observed made us hypothesize that they could be EVs rather resembling

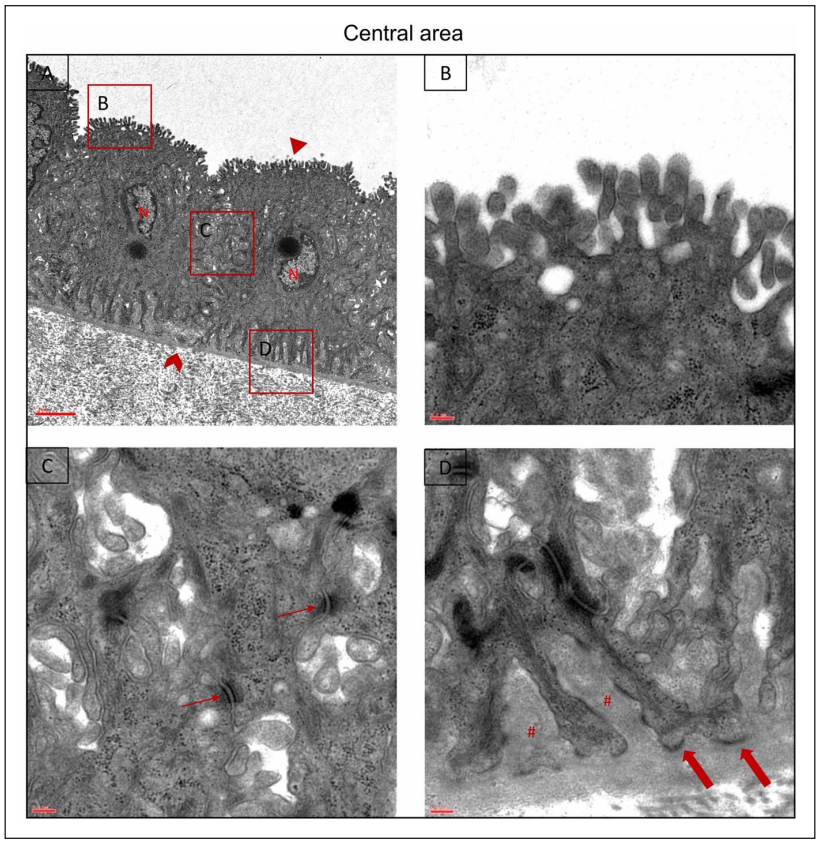


**Figure 1.** Ultrastructure of the amniotic epithelium in the central area. (A) hAECs are in contact with the amniotic cavity (triangle) and the underlying avascular connective layer through a thick basal lamina (arrowhead). Nuclei are euchromatic (N) (3,000×; scale bar: 2 µm). (B, C) The (B) apical and (C) lateral portions are covered by microvilli. Numerous desmosomes are visible (thin arrows) (20,000×; scale bar: 0.2 µm). (D) Cytoplasmic extensions of hAECs enter the basal membrane (#). Emidesmosomes are present (thick arrows) (20,000×; scale bar: 0.2 µm). The most representative sample out of the five different samples is shown. hAECs: human amniotic epithelial cells.

ectosomes (up to 0.5 µm) than exosomes (up to 0.1 µm), according to their size (Fig. 6E). Interestingly, unlike the ultrastructural aspect of exosomes in purified preparations[35], they did not show the typical cap-shaped morphology due to the dehydration process occurring during isolation and enrichment but, instead, they showed a typical round morphology with a different grade of electron density content, probably due to the different type of cargo to release. The vesicles appear membrane bound, are surrounded by an electron-dense granular material, presumably due to the presence of ribosomes (Fig. 6C–E), and form multivesicular bodies whose content is secreted into the extracellular space, via fusion with the plasma membrane (Fig. 6B–E). Finally, to obtain an enriched population of EVs, we isolated epithelial cells from hAM with enzymatic digestion and processed them for TEM. Interestingly, a higher number of the same type of EVs, but in a different phase of development, was observed at the cell surface of the great majority of hAECs (Fig. 6F and G).

Taking into consideration the presence of these EVs only in correspondence of the apical surface of hAECs of the central region, we report in Fig. 7 a schematic cartoon of the ultrastructural features of hAECs from the different regions of hAM.

## Discussion

The potential clinical applications of hAM and hAECs in the field of regenerative medicine have been known in literature since long[41,42]. However, it has yet to be elucidated whether hAM contains different anatomical regions with different plasticity and differentiation potential[3]. Recently, we highlighted for the first time many differences in terms of morphology, marker expression, and differentiation capabilities among four distinct anatomical regions of hAM, demonstrating peculiar functional features in hAEC populations[37,38]. With the present *in situ* ultrastructural characterization, we confirm our previous observations of hAM heterogeneity, and we show for the first time that hAM is

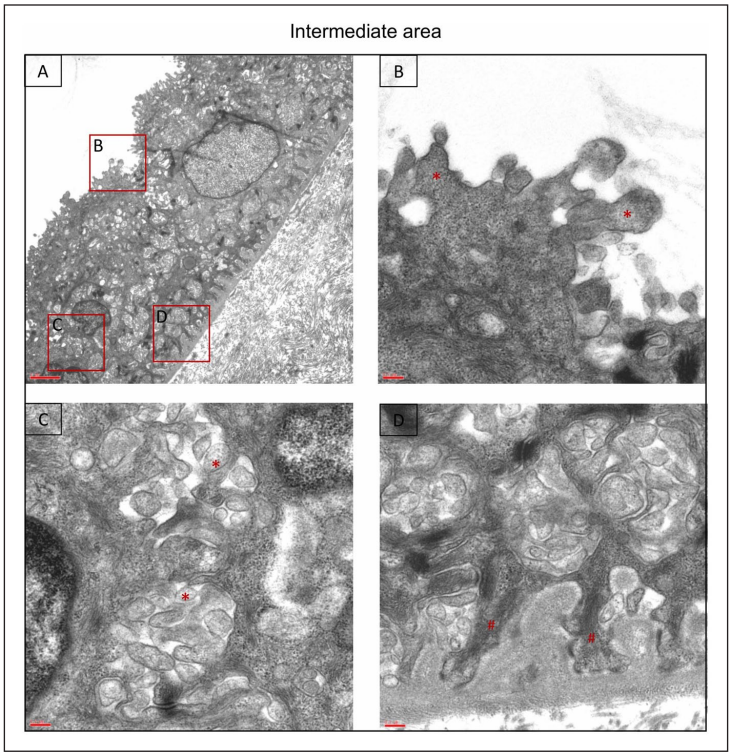


**Figure 2.** Ultrastructure of the amniotic epithelium in the intermediate area. (A) hAECs present a characteristic dome shape (3,000×; scale bar: 2 µm). (B, C) The microvilli in the (B) apical region are thick and short (*) (20,000×; scale bar: 0.2 µm), while they appear more numerous in the (C) lateral region (*) (20,000×; scale bar: 0.2 µm). (D) The hAEC cytoplasmic extensions entering the basal membrane are short and spaced apart (#) (20,000×; scale bar: 0.2 µm). The most representative sample out of the five different samples is shown. hAECs: human amniotic epithelial cells.

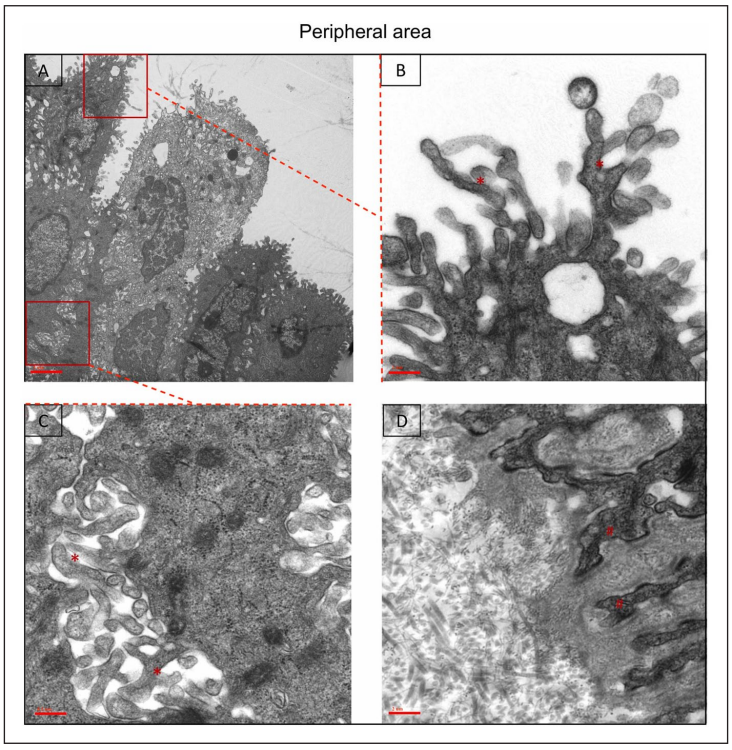


**Figure 3.** Ultrastructure of the amniotic epithelium in the peripheral area. (A) hAECs possess a columnar shape and are organized on multiple layers (3,000×; scale bar: 2 µm). (B, C) The microvilli in the (B) apical and (C) lateral regions are long and thin (*) (20,000×; scale bar: 0.2 µm). (D) The hAEC cytoplasmic extensions appear thin (#) (20,000×; scale bar: 0.2 µm). The most representative sample out of the five different samples is shown. hAECs: human amniotic epithelial cells.

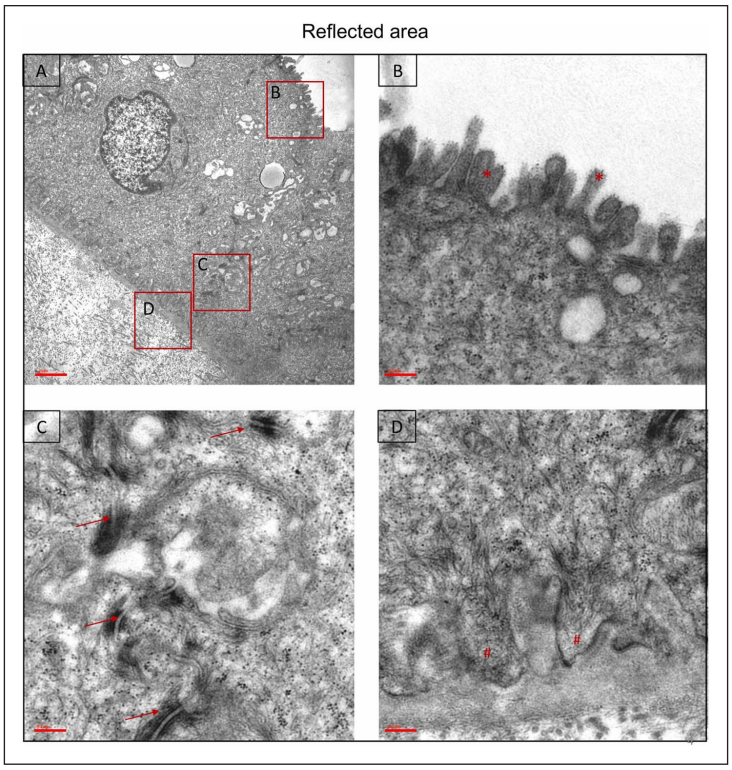


**Figure 4.** Ultrastructure of the amniotic epithelium in the reflected area. (A) hAECs possess a cuboidal shape and a smoother surface (3,000×; scale bar: 2 µm). (B, C) The microvilli in the (B) apical region are short, while they are few in the (C) lateral area. Numerous desmosomes are also visible (thin arrows) (20,000×; scale bar: 0.2 µm). (D) The hAEC cytoplasmic extensions are short and thick (#) (20,000×; scale bar: 0.2 µm). The most representative out of the five different samples is shown. hAECs: human amniotic epithelial cells.

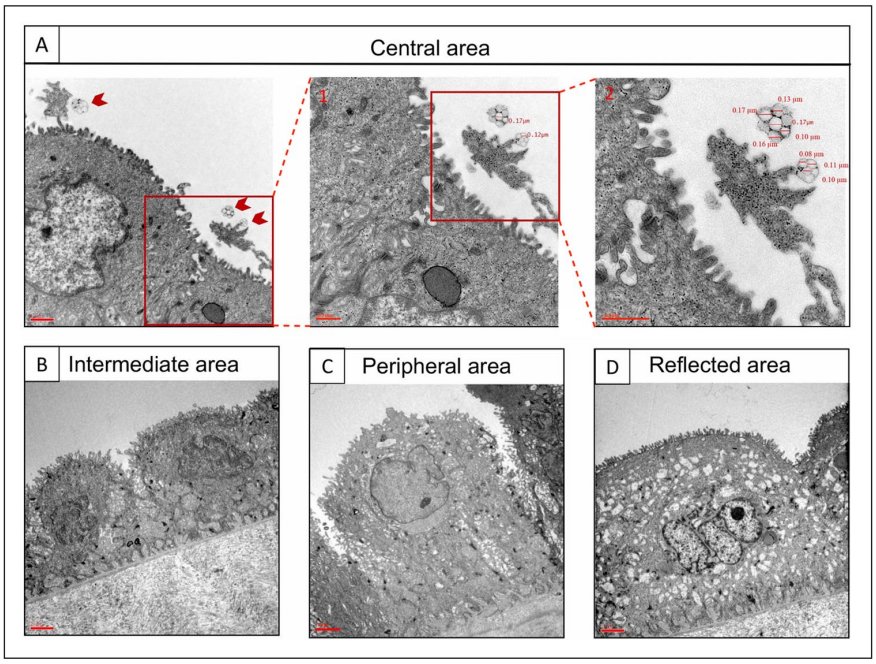


**Figure 5.** Ultrastructural detection of extracellular vesicles (EVs) secreted by the central area of hAM. (A) The figure shows a detail from the central area at different magnifications, highlighting the presence of EVs near the apical surface of the epithelial cell (A: 7,000×, scale bar: 1 µm; A1: 12,000×, scale bar: 0.5 µm; A2: 20,000×, scale bar: 0.5 µm). (B–D) The presence of these structures was not detected in the other regions of hAM (7,000×, scale bars: 2 µm). The most representative sample out of the five different samples is shown. hAM: human amniotic membrane.

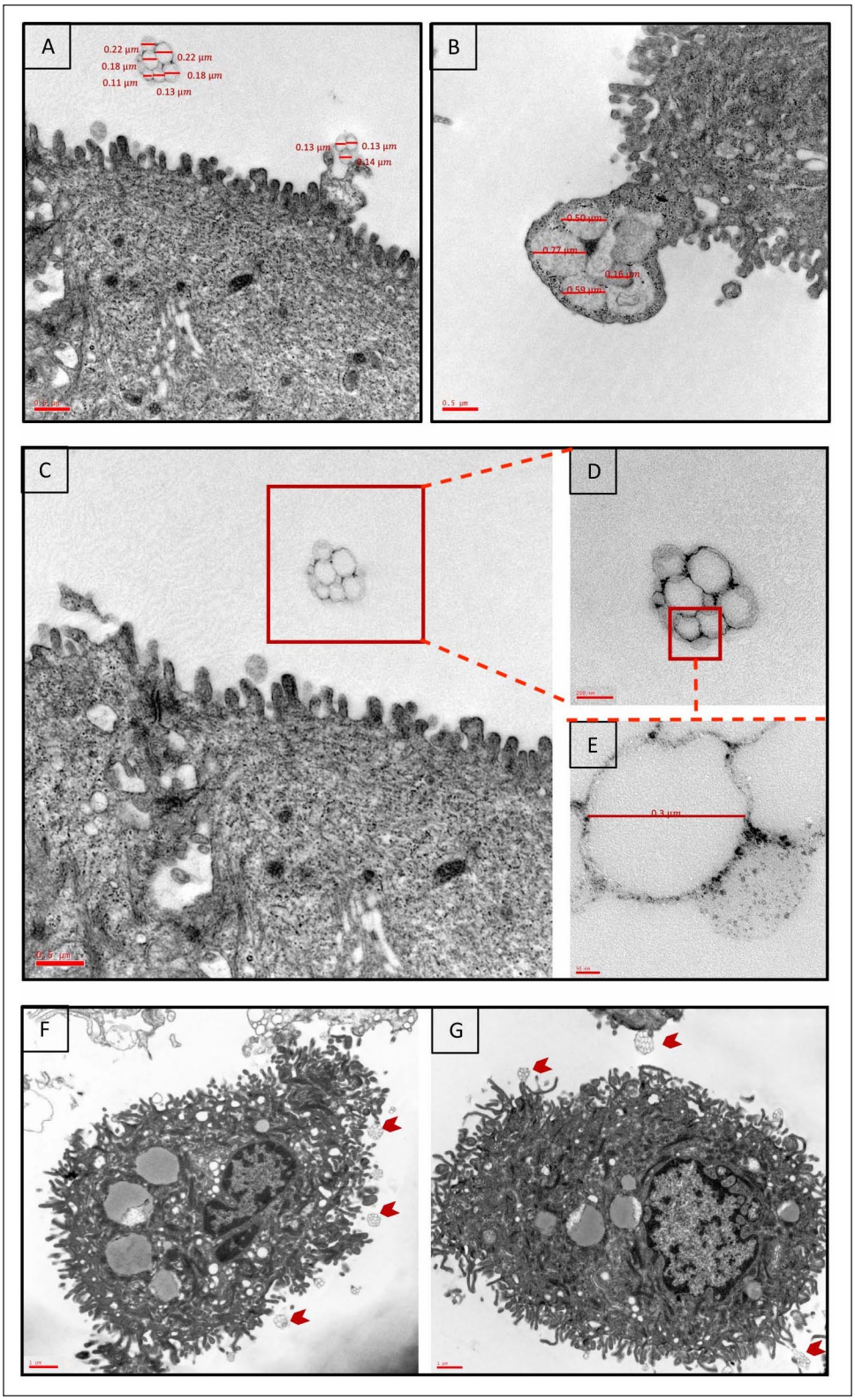


**Figure 6.** Ultrastructural detection of extracellular vesicles (EVs) secreted by the central area of hAM. (A–B) The figure shows a group of microvesicles identified by their range of size between 100 and 1,000 nm. They seem to bud from the apical surface of the cytoplasm (12,000×, scale bar: 0.5 µm). (C–E) A group of EVs secreted by the central area of hAM at different magnifications is shown (12,000×; 20,000×; 50,000×; scale bars: 0.5 µm, 200 nm, 50 nm). (F–G) The figure shows several multivesicular bodies around the apical surface of hAECs after their enzymatic separation from the basal lamina (arrowheads) (3,000×, scale bars: 1 µm). The most representative samples out of the 10 different samples are shown. hAM: human amniotic membrane; hAECs: human amniotic epithelial cells.

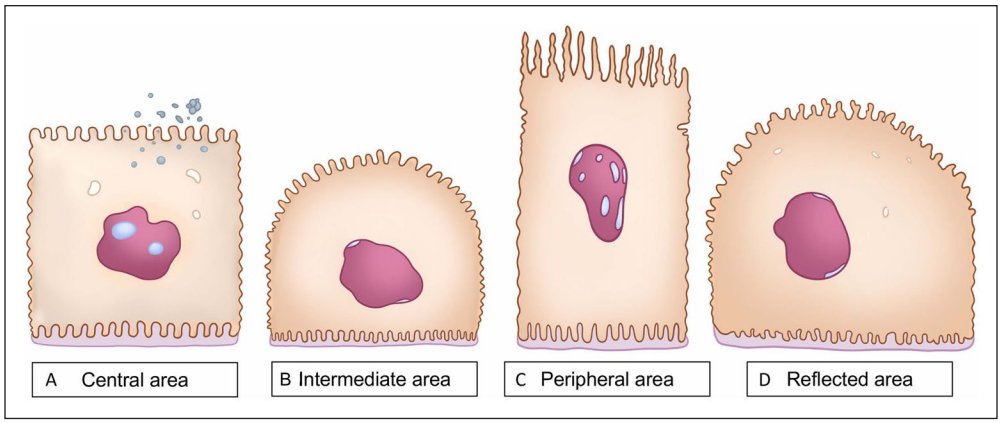


**Figure 7.** Schematic cartoon of hAEC structural features from the different regions of hAM. (A) Central area: cells are mainly cuboidal, characterized by short and thick microvilli distributed at the apical, lateral, and basal surface; round aggregates of microvesicles are visible near the apical surface. (B) Intermediate area: cells show a dome shape, numerous microvilli at the basal surface compared with the central area. (C) Peripheral area: cells present columnar shape, long and thin microvilli. (D) Reflected area: cells show a dome shape with squat microvilli. hAECs: human amniotic epithelial cells; hAM: human amniotic membrane.

able to produce EVs in a heterogeneous manner. In the past several years, two different areas of hAM have been identified based on their peculiar characteristics: the placental amnion and the reflected amnion, respectively[43]. Placental amnion has been shown to possess a higher metabolic activity and significantly lower reactive oxygen species content when compared with reflected amnion, suggesting diverse cell differentiation capacities between the two regions[44–46]. Only few data reported histological and marker expression differences leading to hypothesize that hAM and hAECs are biologically heterogeneous[47–53]. To find ultrastructural studies, we must go back to 1965 when Thomas[48] reported for the first time that the placental and reflected portions of hAM were characterized by epithelial cells with different ultrastructural features, and possible differences in secretory activity were also hypothesized. In this regard, it is well known from literature that the hAM is involved in absorption and/or secretion processes during both early and late pregnancy with the involvement of MVs and placenta-derived exosomes[54]. However, exosome studies are usually carried out using preparations obtained with a wide range of methods, including centrifugation of biological fluids or cell secretome followed by ultracentrifugation as well as ultracentrifugation in a density gradient[33]. It should be noted that these methods do not allow the obtainment of homogeneous pure preparations, thus leading to high data variability in exosome molecular composition and lacking important information on localization and secretion modalities[34]. The results of this study showed for the first time that hAM and hAECs are characterized by peculiar aspects and differences in terms of ultrastructure and secretion capabilities among the central, intermediate, peripheral, and reflected areas. Indeed, hAECs from the different regions of hAM are heterogeneous both in shape and cytoplasmic content. In the central and reflected areas, the cells are mainly cuboidal, whereas in the intermediate area, they have a dome shape and in the peripheral area, they are taller and with a columnar aspect (Fig. 7). Several differences in the microvilli aspect both on the surface and in the lateral compartment of hAECs can be observed. They appear short and thick in hAECs present in the central and intermediate areas, while in hAECs present in the peripheral area, they are longer, thinner, and more numerous. In the cells present in the reflected region, they are similar with those of the cells belonging to the central area. Interestingly, numerous gaps and lateral microvilli in the intercellular space between the cells are present in the central, intermediate, and peripheral areas. In fact, it is well known that thanks to the presence of microvilli, the amniotic epithelial cells exert secretory function and that these microvilli probably take part in intracellular and transcellular transports and communication[55]. Remarkably, the reflected area presents peculiar differences compared with the other areas. The apical microvilli features are similar to those present in the central area, while lateral microvilli and gaps in the intercellular space are less numerous and many desmosomes can be appreciated. Several differences can also be observed at the basal surface where the hAECs are anchored through cytoplasmic extensions which enter the basal membrane. They appear longer in the central and peripheral areas, while in the intermediate and in the reflected areas, they are shorter and spaced apart. These findings demonstrate that the amniotic epithelium is not only heterogeneous but also constantly in evolution. The presence of microvilli and gap spaces in the intercellular region of hAECs in the central, intermediate, and peripheral areas highlights their role in secretion and absorption activities. In contrast, the reflected area appears in a "quiescent state" compared with the other regions, due to the shorter microvilli, the few intercellular spaces, and the short and underdeveloped cytoplasmic extensions in the basal

membrane. Furthermore, in the reflected area, numerous desmosomes can be appreciated compared with the other regions. All these findings suggest that hAECs in the central, intermediate, and peripheral areas are more "stressed" than the cells in the reflected area. They appear detached from each other, thanks to the presence of lateral microvilli and gap spaces, and this could be probably due to mechanical tension and continuous contact with the amniotic fluid. In contrast, the presence of desmosomes in the reflected area demonstrates closest intercellular bonds between hAECs. Interestingly, beside the differences in secretory granule content already reported in a recent paper of our lab[37], we observed some groups of EVs in the apical surface of hAECs belonging to the central area (Fig. 5), revealing that this region is particularly involved in the fetomaternal communication being closer to the umbilical cord. Digital morphometric measurements of the structures observed were performed, and according to the results obtained, we identified them as MVs and multivesicular bodies (Fig. 6). Unlike what has been reported in literature, we identified these structures also in the extracellular space in a different phase of development in relationship with the extent of the release of the secretory product. Based on our morphological observations, we can argue that the finding of isolated exosomes can be an artifact due to the complex procedures commonly employed to enrich them from biological liquids or secretome[35]. Moreover, in literature, there is no mention of the precise area of hAM capable to produce and release exosomes because data available concern the whole AM[26]. Thanks to TEM analysis, we were able to detect and study MVs *in situ*, avoiding limitations connected to the methods classically used to isolate and study exosome-like structures. In this regard, the ultrastructural analysis of hAECs enzymatically isolated from the basal membrane revealed the presence of an increased number of MVs, indicating that the mechanical and chemical stresses applied to isolate hAECs are probably responsible for an overproduction of MVs, in comparison with hAM prepared for *in situ* analysis, leading to their consequent overestimation in terms of number and content analysis.

In conclusion, our data show for the first time the ultrastructural heterogeneity of four different anatomical regions of hAM, not only in terms of morphological features but also with respect to secretory activity and different capacity to produce and release MVs, although further studies are needed to characterize them. These findings should be considered to increase efficiency of hAM applications within a therapeutic context.

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## Authors' Contributions

MB performed the experimental study, analyzed the data, and wrote the paper. LC did the morphometric analysis. FP and GS performed the isolation of amniotic membranes and prepared the figures. OS and SS performed bibliographic research. FG and AS provided materials and technical support. RDP and OP supervised the study and analyzed the data. RDP designed the study and revised the paper. All authors read and approved the final manuscript.

## Data Availability Statement

The data are available upon request from the corresponding author.

## Ethical Approval

Ethical approval was obtained from the Ethics Committee of the G. d'Annunzio University of Chieti-Pescara, Italy, and the local ethical committee "Comitato Etico Provinciale di Brescia," Italy (protocol number: 191/6.2.17 Tit. III Cl. 13; number NP 2243, January 19, 2016).

## Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Ethics Committee of the G. d'Annunzio University of Chieti-Pescara, Italy.

## Statement of Informed Consent

Written informed consent was obtained from the patient(s) for their anonymized information to be published in this article.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Supplemental Material

Supplemental material for this article is available online.

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