

PATTERN OF M30 IMMUNOHISTOCHEMISTRY DISTRIBUTION IN NORMAL HUMAN PLACENTAL VILLI IN RELATION TO ECCENTRIC UMBILICAL CORD INSERTION

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ABSTRACT

Background: The growth and development of human fetus is depending on the placenta, thus any alterations in the normal development, function and morphology of the placenta and umbilical cord could result in adverse outcome on the pregnancy and deleterious effects to both mother and child. **Materials and methods:** 20 normal term placentas with eccentric umbilical cord insertion (9 placentas were included in IHC) were collected from none smoker healthy pregnant women (have no: hypertension, diabetes, preeclampsia and any other chronic diseases or gynecological problem), who underwent normal vaginal delivery. The samples (placentas) were directly collected after being expelled from the uterus and were examined to ensure that they have an eccentric umbilical cord insertion. In this study, M30 Cytodeath was used for assessing the immunohistochemical reactivity of M30 in trophoblastic villous of placental tissues to investigated if the programmed cell death (apoptosis) is related to the eccentric umbilical cord insertion. The analysis of the results of immunohistochemical study was achieved by applying Aperio positive pixel count algorithms program. **Results:** In this study, the immunohistochemical reactivity for the M30 Cytodeath was seen mainly in the placental trophoblastic villi of term human placenta and the four zones of placental villi that have been studied showed the same pattern of M30 immunohistochemical reactivity, while the reactivity of the M30 Cytodeath was poor in the core villi and the syncytial knots, and the differences in the data of M30 immunohistochemical expression, showed that the programmed cell death (apoptosis) detected in placental trophoblastic villi was not related to the eccentric umbilical cord insertion, because the mean positivity in the four zones of term human placenta, showed statistically non-significant differences (p value > 0.05).

KEYWORDS: Term placenta, eccentric umbilical cord insertion, placental villi, M30 CytoDeath, immunohistochemistry, and apoptosis.

INTRODUCTION

Placenta is a miracle organ essential for the development of the fetus in the uterus (Akhlaf *et al.*, 2012), responsible for the transfer of nutrients (Hay *et al.*, 1997), gaseous exchange, synthesise important hormones and protect the fetus from the mother's immune system (Ni and Mao 2011). Normal development of the placenta depend upon extravillous trophoblast and the remodeling of maternal spiral arteries invading the maternal decidua to furnish placental stability and efficacious utero-placental blood flow (Cartwright *et al.*, 2010). With further development placental branched chorionic villi furnish a wide surface area in which elements (e.g., oxygen and nutrients) are reciprocated through the delicate placental membrane mediated fetal and maternal circulations (Keith *et al.*, 2016).

At approximately 12 to 13 days after fertilization, proliferation of the cytotrophoblasts into the

syncytiotrophoblasts is seen and subsequent blind-ended branches protrude into the lacunar space, forming a primary villi which marks the growth of villous tree of the chorion (Bernischke *et al.*, 2006). In human placenta, the syncytiotrophoblast make direct attachment to the blood inside maternal intervillous spaces (Mori *et al.*, 2007). The trophoblast and extra-embryonic mesoderm develops into the vascular chorionic plate and the villous tree (Hacker *et al.*, 2004). Throughout first trimester period, growth of configuration villous tree is fast, but third trimester period reveals reduced villi formation and maturation (Mayhew, 2001). As gestation advanced the villous trophoblasts thinnest irregularly, and the cytotrophoblasts regressed, alterations explained as an aging structure nature compare with immature intermediate villi that predominance in first trimester period (Mayhew and Simpson 1994). In fact, the placental development and synthesis of DNA stopped around 36th week of gestation, and further increase in

placental volume result in accretion in the size of cells rather than increasing numbers of the cells (Iverson and Farsund, 1985).

The umbilical cord also referred to as Funiculus umbilicalis or birth cord. It is the structure that connects placenta to the fetus (Ozdemir *et al.*, 2007). Umbilical cord considered as a part of the fetus genetically and physiological aspects, providing the nutrients (Predanic, 2009), oxygen, and fluids necessary for life in utero (Ferguson and Dodson 2009), and eliminates waste products (Malden, 2009). The umbilical cord is attached to the fetal side, the chorionic plate of the placenta, where the vessels branch into a meshwork enveloped by a delicate layer of cells (Chang *et al.*, 2012). Purposefully, most important role of umbilical cord is to facilitate fetal development until delivery; therefore, the cord needs not to attach which in turn demands a specialized anatomy of its insertion to both the fetus and the placenta. Failure of such an attachment would cause fetal demise (Collins, 2002).

In fact, the normal attachment of umbilical cord is at the central site of the placenta a far from its edge, but occasionally it exhibits an eccentric attachment. Other less common types of insertion are marginal, velamentous and furcated umbilical cord (Singh and Pal, 2009). However, whether the insertion of the umbilical cord to placenta is central or eccentric, it is considered as normal, and found in more than 90% of all cord insertions into the placenta (Pathak *et al.*, 2010), that have no clinical importance, while marginal insertion may be further susceptible to laceration of the vessels and suggested to link to the IUGR, still birth and neonatal demise (Heifetz, 1996).

Apoptosis or cell death, is an essential active process that plays a crucial role in the growth and development of organisms as well as in the maintenance and regulation of cells in tissues under physiological and pathological circumstances (Leist and Jaattela, 2001). Apoptosis is well-recognized and probably the most frequent form of programmed cell death (Chan *et al.*, 1999). In general, most pathways of apoptosis involve a sensor which detects the (death-inducing signal), that triggers programmed cell death by a signal transduction network resulting in activation of a cascade of intracellular proteases named (caspases), the execution machinery that has a responsibility to carrying out the cell death process. The last step is the elimination of the dead cells, through engulfment of cell corpses by other cells, usually macrophages (Jirina *et al.*, 2003). Apoptotic cells are highly active cells with changes observed in the nucleus and cytoplasm (Abumaree *et al.*, 2012; Corrêa *et al.*, 2008). In fact, it is a controlled and energy depending process, hence cell components involving DNA broken down and cell organelles fragment are condensed and packaged inside the cell membrane, forming a distinct dense apoptotic bodies (Fadok *et al.*, 1992).

Furthermore, apoptosis is increased with advanced age, possibly in response to increasing DNA damage, increasing reactive oxygen species (ROS), a decrease in the GH-IGF axis which regulates cellular proliferation and differentiation (Bonefeld and Moller 2011; Sharp *et al.*, 2010), and telomere shortening (Bewley *et al.*, 2009). However, cell death by apoptosis is considered an essential regulator of the cell numbers in the normal tissues and many diseases (Bantel *et al.*, 2001), such as alcoholic hepatitis (Natori *et al.*, 2001), and viral chronic hepatitis (Bantel *et al.*, 2001). Apoptosis plays an essential role in multiple aspects of placentation, from the insertion of blastocyst to the endometrium and its subsequent implantation (Galan *et al.*, 2000) to maternal-placental tolerance (Abrahams *et al.*, 2004), as the placenta undergoes tissue alterations, that distinguished by a functional trophoblastic cells loss. Selective removal of aging trophoblastic cells by more young trophoblasts actually occurred without influencing the neighbor cells (Mayhew, 2001), the structural maintenance of placental tissues and its specific function includes cellular alterations that is highly regulated depending on a distinct balance which involves the proliferation and differentiation of the cells, as well as cellular death (Benirschke and Kaufmann, 2000). As a normal process of trophoblastic turnover apoptosis, releasing of apoptotic substances does not induce any responses even inflammatory responses to the mother (Huppertz and Gauster, 2011).

In 1996, it was the first scientific study that has been described trophoblastic apoptosis within normal placental tissues. Although observations of histological features regarding trophoblastic apoptosis were described prior to this year (Smith *et al.*, 1997). Meanwhile, many other investigations have been improved that the syncytiotrophoblast undergoes degeneration and has an apoptotic features in the first trimester too (Chan *et al.*, 1999). Components of both pathways of apoptosis (intrinsic and extrinsic) have been illustrated in trophoblasts, and their expression revealed alterations up to gestational period associated with changes in villous development (Huppertz and Kingdom, 2004). The characteristic features of apoptosis include: cell shrinking, membrane alterations of the cells, chromatin condensation, nuclear fragmentation, chromosomal DNA fragmentation, creation of apoptotic bodies, breakdown of mitochondria, and releasing of cytochrome c (Aplin, 2010). These cellular changes result largely from the activation of executioner or effector caspases, which is a family of (cysteine- aspartate proteases) (Jocelyn, 2010), which is a key player in apoptosis (Chowdhury *et al.*, 2006), and able to cleave and activate a variety of pro-apoptotic proteins as part of a cascade of events that culminate in cell death. Apoptotic process is normally completed within 24 hours (Suzuki *et al.*, 2001). However, caspases are divided into initiator caspases (caspase-2, -8, -9, -10) and effector caspases (caspase-3, -6, and 7) (Li and Yuan, 2008).

Fuentes and Salvesen 2004 cited that “once activated, these members function primarily in the cleavage and activation of downstream effectors caspases, thereby initiating what is known as the caspase cascade”. In fact, increased mitochondrial permeability causes membrane pore formation and cytochrome c leaks towards the cytosol (Figure 1) (Li *et al.*, 1997). Immediately after the releasing of cytochrome c, it binds to the apoptotic protease activating factor-1 (Apaf-1) and ATP, subsequently, it binds to pro-caspase-9 to form a protein complex called an apoptosome which cleaves the pro-caspase to caspase-9, (the active form of pro-caspase), which then activating the effector caspase-3 (Dejean *et al.*, 2006).

The initial stages of apoptosis occur in the cytotrophoblast layer, where expression of (initiator caspase 8) is demonstrated, and cleavage of a-fodrin (a cytoskeleton protein), as well as by the externalization of phosphatidylserine (an aminophospholipid) starting from inner leaflets of plasma membrane to its outer margin (Huppertz *et al.*, 1999). Evidences of caspases 3, 8 and caspase 9 activities within villous trophoblasts, with procaspase 3, were localized within cytotrophoblast and syncytiotrophoblast (De Falco *et al.*, 2005). The increasing up regulation of caspase-8, and caspase-3 expression in placental tissues throughout normal gestation was observed to involve in the angiogenesis suppression and development of placental tissues (Sokolov *et al.*, 2009). Different stages of the cascades were correlated with differentiation, syncytial fusion, and degeneration of villous trophoblast (Black *et al.*, 2004).

The trophoblastic apoptosis events increased as gestation progresses and by the third trimester as much as 3 grams of syncytial material is shed into the maternal circulation a day (Huppertz *et al.*, 1998). Extrinsic apoptotic signals like tumor necrosis factor- α (TNF) and interferon- γ are produced by cytotrophoblast and probably essential for induction of apoptosis machinery in the immune cells to prevent the placental recognition (Chen and Goeddel, 2002).

Cytotrophoblast and syncytiotrophoblast, expressed TNF receptors, as well as Fas and Fas ligand, along with TNF-related apoptosis-inducing ligand (TRAIL) (Bai *et al.*, 2009), all of which may have a crucial role in apoptotic process as well as in immune regulation (Straszewski *et al.*, 2005). Murphy and his colleagues 2000, cited that, following activation of TNF receptors (TNF-R1) and Fas a balance between the anti-apoptotic: Bcl-XL and Bcl-2, which are members of Bcl-2 family and proapoptotic: BAX, BID, BAK and BAD, is establishing, these proapoptotic members are essential and eventually required to enhance the permeability of mitochondrial membrane for the releasing of cytochrome c and the mitochondrial protein SMAC, the caspase activators. In fact, a proapoptotic BAX protein is rarely expressed in cytotrophoblast and it is undetectable in syncytiotrophoblasts, but it was distinguished in

connective tissue as well as in the perivascular cells inside the villous core (Ratts *et al.*, 2000).

The filamentous structures, cytokeratin are existed in the cell cytoplasm as well as nucleus. The cytoskeletal network integration created by the microfilaments, intermediate filaments (IF) and microtubules (Barak *et al.*, 2004). In the developing placenta, cytokeratin 18 (K18) is expressed in all trophoblast cells derived from the differentiation of trophoblast (TE) until term (Magin *et al.*, 1998). K18 considered as the major cytoskeletal proteins of epithelial cells. It comprises ~ 5 % of total cell protein (Thompson, 1995).

K18 is cleaved by caspases, liberating a neo-epitope (M30) that is specifically recognized by the M30 CytoDEATH™ monoclonal antibody. Specific proteolytic cleavage of K18 is an event taking place before disruption of membrane asymmetry and induction of DNA strand breaks. Numerous studies confirm that M30 CytoDEATH™ antibody detects only apoptotic but not viable or necrotic cells (Walker and Quirke, 2001). The capacity of M30 CytoDeath™ antibody in flow cytometry and immunohistochemistry studies to distinguish between necrotic and apoptotic epithelial cells has been verified in several disease entities. Consequently, M30 CytoDeath™ antibody represents a unique tool for easy and reliable determination of apoptosis from very early until well advanced stages in single cells and tissue sections of epithelial origin (Grassi *et al.*, 2004).

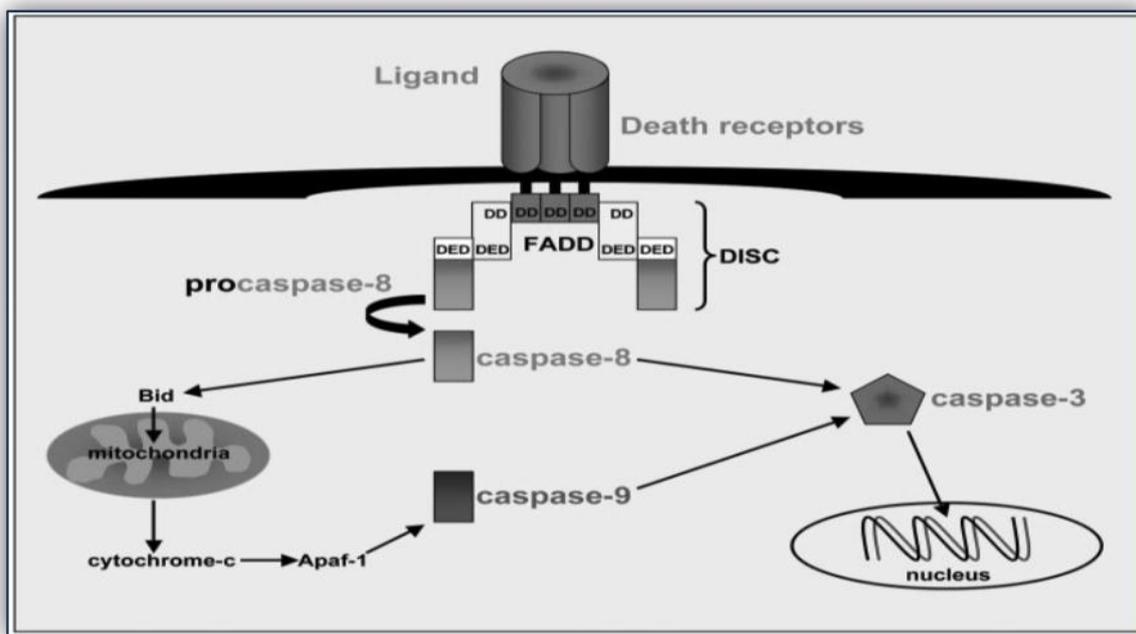


Figure 1: Both pathways- the intrinsic and extrinsic depending on the activated stimuli, apoptosis begins by one of two recognized pathways; the intrinsic (mitochondrial) pathway or by the extrinsic pathway which also known as death receptor-mediated pathway. Shawn *et al.*, 2005.

MATERIALS AND METHODS

The samples included in this study are normal human placenta taken from Al-Alwaiya maternity teaching hospital. 20 normal term placentas with eccentric umbilical cord insertion were collected (nine placentas were included in IHC) from none smoker healthy pregnant women (have no: hypertension, diabetes, preeclampsia and any other chronic diseases or gynecological problem), who underwent normal vaginal delivery. The samples (placentas) were directly collected after being expelled from the uterus and were examined to ensure that they have an eccentric umbilical cord insertion. The gestational age of the pregnant mothers was determined by history taken from those women prior to delivery to ensure term pregnancy which is consider to be 38-40 week from the first day of the last normal menstrual.

After the placenta being examined, to ensure that they have an eccentric umbilical cord insertion, the placental membranes were trimmed off and the umbilical cord cut to within 2 cm of its insertion. Then the largest diameter of the placenta, using its fetal side, that passing through the area of cord insertion was excised in a form of strip with 3 cm width. Later on, this strip was sectioned into 4 equal zones (named A, B, C and D) as shown in (figure 2); then from the middle part of each area a tissue sample (with dimensions of about 1 x 1cm) was excised. Only the maternal portion of those tissue samples was included in this study. The placental tissue samples after being excised previously from the middle area of each region (1x1) cm² was fixed for 6 hours in 10% neutral

buffered formalin (PH 7.3) then these samples were further cut into smaller sections of about (5x5x7) mm³, these sections were further fixed for 18 hours, resulting in a total fixation period of about 24 hours.

M30 CytoDEATH™ (ROCHE, Germany) mouse monoclonal antibody (Clone M30) was is used in this study for detection of early apoptosis in placental trophoblastic cells.

Additional marker; CK18 (Pathnsitu, USA) primary antibody was used in this study to demonstrate the CK18 distribution in the placental villi and was considered as a control. Furthermore, sections of human colonic adenocarcinoma were used as positive and negative control. However, CK18 is cleaved by caspases, liberating a neo-epitope (M30) that is specifically recognized by the M30 CytoDEATH™ monoclonal antibody. Specific proteolytic cleavage of CK18 is an event taking place before disruption of membrane asymmetry and induction of DNA strand breaks.

The lyophilized M30 CytoDeath™ was dissolved using incubation buffer (Tween Phosphate Buffer Saline (TPBS) which was prepared by dissolving 1gm of Bovine serum albumin (BSA) into 100 ml of phosphate buffer saline (PBS) with 0.1 ml of tween 20) and detected by using polyExel HRP/DAB detection system (Pathnsitu, USA). While the (phosphate buffer saline) PBS was prepared by dissolving (8 gm NaCl, 0.2 gm KCL, 1.424 gm Na₂HPO₄*2H₂O and 0.2 gm HH₂PO₄) in 1000 ml distilled water (Suvarna *et al.*, 2013). The

procedure for immunohistochemical reactivity was carried out according to Sigma and Pathnsitu protocols (with some modifications achieved for enhancement).

The positive charge slides holding the sections treated with M30 Cytodeath antibody and ck18 antibody were examined using the 3DHISTECH scanner (Panoramic Desk, Hungary) with its 3DHISTECH software which already installed on an attached computer. Random samples of fields (5 fields), from the Immunohistochemical slide were captured, the immunoreactivital reactivity of placental tissue sections were estimated by using Aperio positive pixel count

algorithms program (from Aperio Image Scope Software V.12 (Aperio Technologies Inc, USA) downloaded from the official website. It was selected to analyze the digital images. In addition, the statistical analysis of data was performed using IBM SPSS software version 24 installed on personal computer. The statistical differences between the means of the positivity for the immunohistochemical reactivity in studied placental zones were analyzed using the analysis ANOVA test. Another statistical difference for the means of the immunopositive reactivity was done between zone A group and with the mean different of each of the following zones (B, C, and D) groups using an independent samples t test.

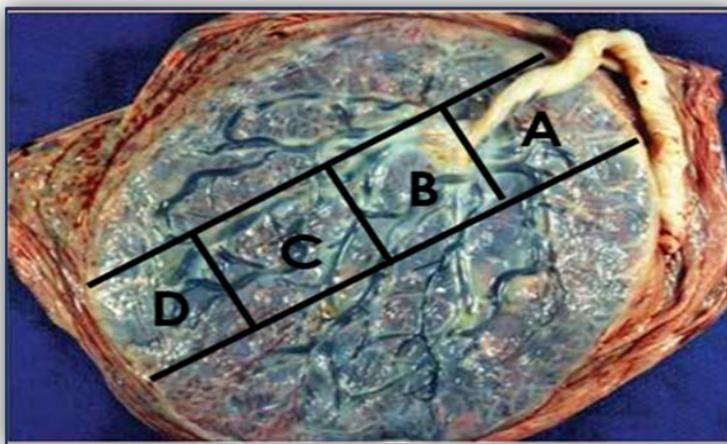


Figure 2: term placenta with a schematic diagram showing the placental strip with its 4 zones (A, B, C, and D). Derick Mussen Healthcare. (2017). Retrieve from: <https://www.mussenhealth.us/dividing-membranes/i.html>.

RESULTS

Immunohistochemical reactivity of M30 in trophoblastic villous

The M30 immunostaining expressed mostly in the syncytium and poorly in the cytotrophoblast, the four zones show the same pattern of M30 immunohistochemical reactivity, while the reactivity of the M30 Cytodeath was poor in the core villi and the syncytial knots (Figures 3, 5, 7, 9, 11, 12, 13, and 14).

M30 cytodeath immuno-staining was applied to sections of adenocarcinoma of human colon as positive and negative controls (Figure 15) and (Figure 17). Negative control was also performed to the placental tissue by applying only the detection kit without the primary antibody (M30 Cytodeath) (Figure 19).

Another marker (CK18 (pathncyto, USA) primary antibody) was used, as another control, to illustrate the pattern of immunoreactivity in the trophoblastic cells of the term placenta. The immunoreactivity of this marker, CK18, was expressed on both cytotrophoblast and syncytium (Figure 21).

Table1 and (Figure 23) showed the mean positivity of M30 immune reaction in the trophoblast of term human

placenta of the studied zones, which reflects the apoptosis in the trophoblast of the placental villi. The mean positivity, of immunohistochemical reactivity of M30, showed the highest value in sections obtained from zone A (3.972 ± 0.43964), while the lowest value was recorded in sections of zone B (2.7696 ± 0.44111). Furthermore, the mean positivity of sections obtained from the zones C and D were (3.1647 ± 0.46386) and (2.7993 ± 0.41830) respectively.

However, analysis of these differences in the mean positivity in the four zones of term human placenta, using ANOVA test, showed statistically non-significant differences (p value > 0.05).

According to table2, the comparison between the mean number of M30 apoptosis trophoblastic villi in zone A group having (3.9720 ± 2.94922) and zone B group that have (2.7696 ± 2.95904) were statistically non-significant using the independent samples t test, (p value = **0.057**).

While the comparison between the mean number of M30 apoptosis trophoblastic villi in zone A group having (3.9720 ± 2.94922) and zone C group that have ($3.1647 \pm$

3.11169) were also statistically non-significant using the independent samples t test, (p value = 0.210) (Table3).

In addition, the comparison between the mean number of M30 apoptosis trophoblastic villi in zone A group having (3.9720 ± 2.94922) and zone D group that have (2.7993 ± 2.80605) were also statistically non-significant using

the independent samples t test, (p value =0.057) (Table 4).

In other words, the mean different between zone A group and with the mean different of each of the following zones (B, C, and D) were statistically non different.

Zone A Field

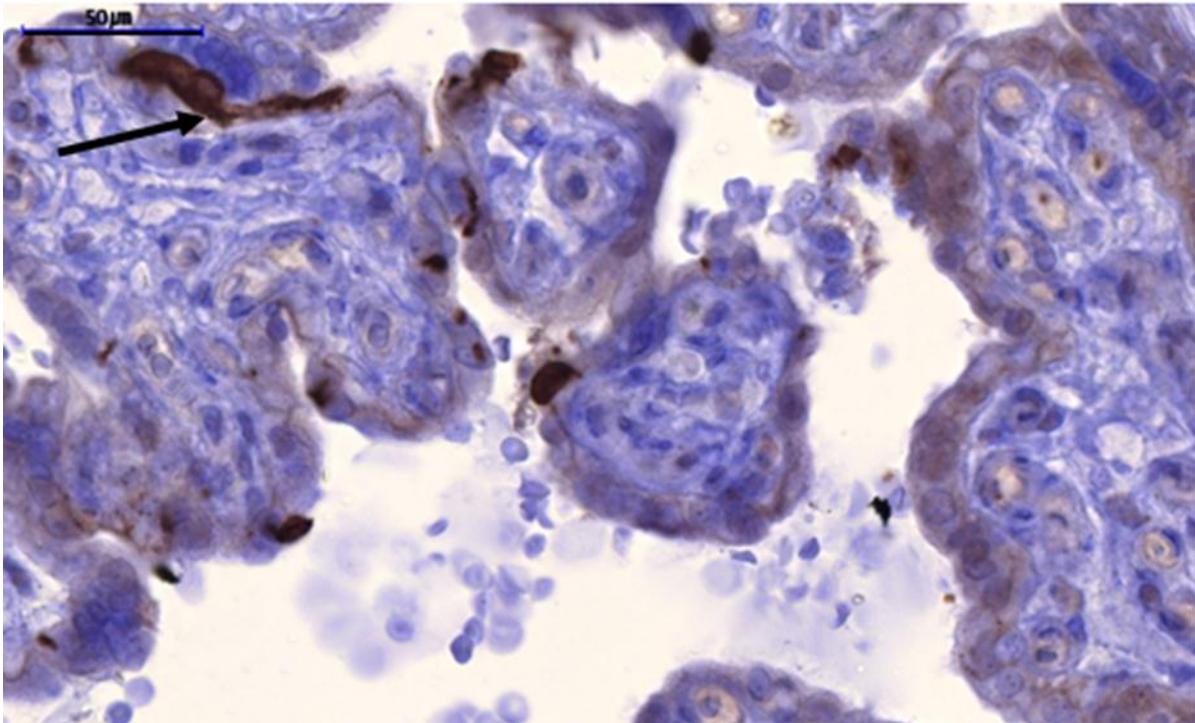


Figure 3: illustrate the M30 Cytodeath reaction (Black Arrow) 40X.

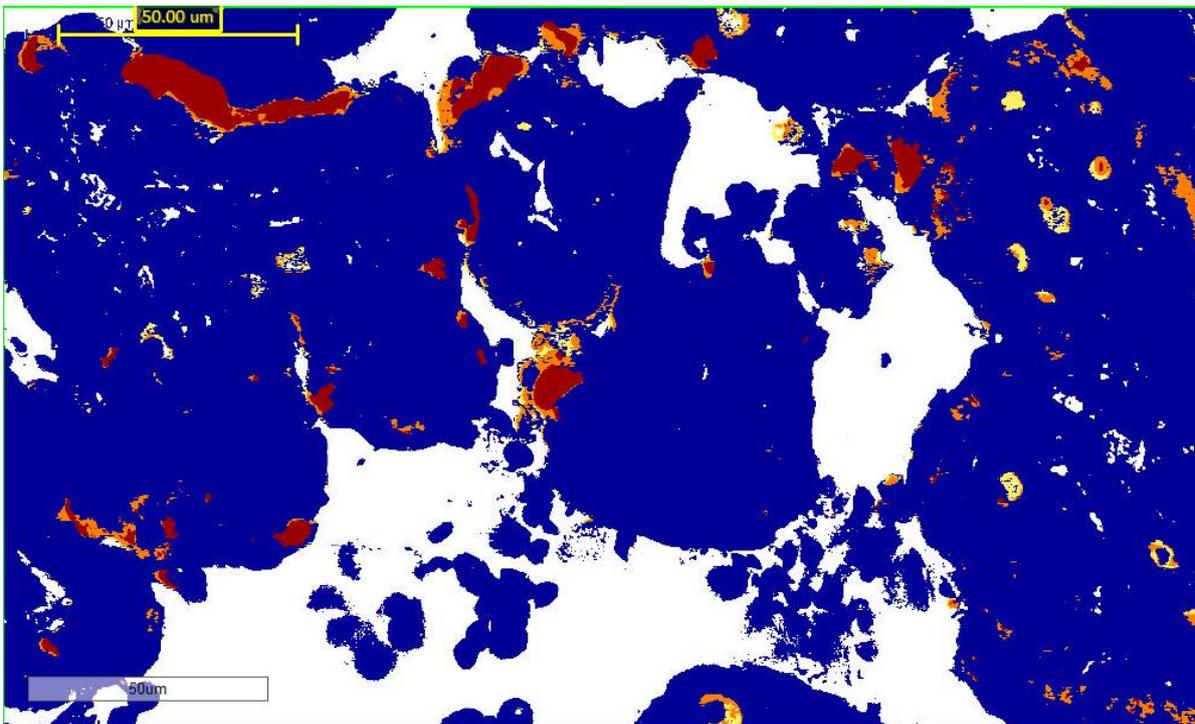


Figure 4: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

Zone B Field

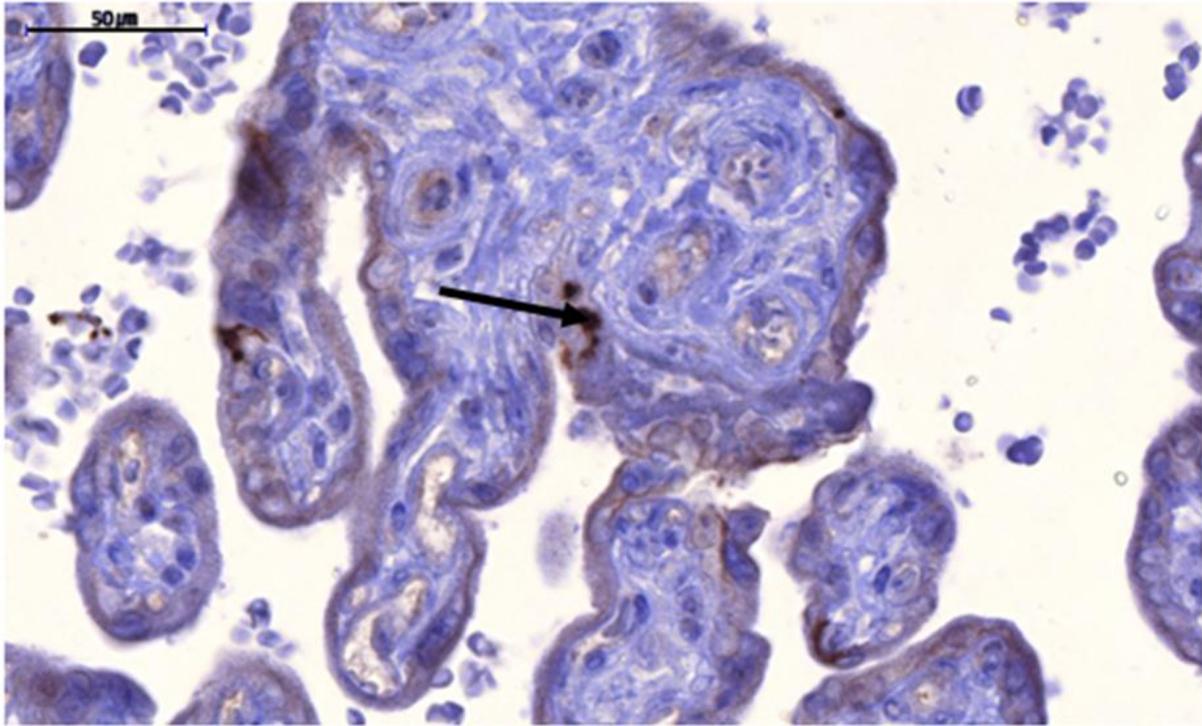


Figure 5: illustrate the M30 Cytotoxicity reaction (Black Arrow). 40X.

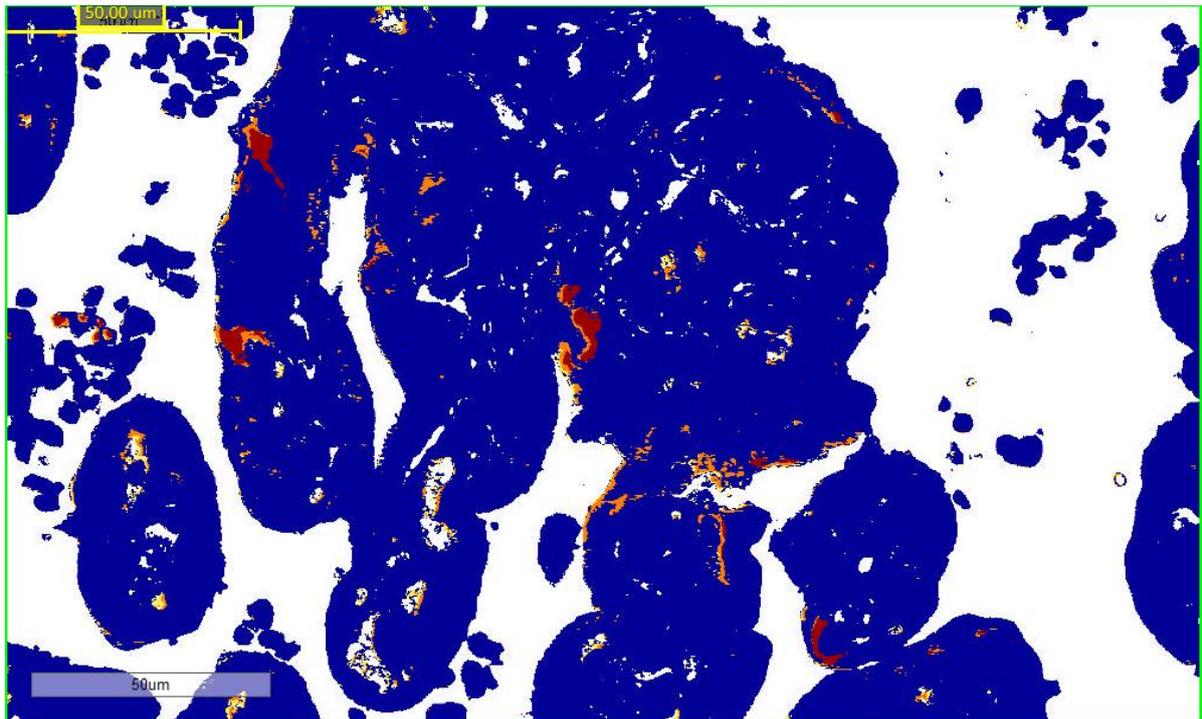


Figure 6: illustrate the color deconvolution resulted from Aperio image scope software analysis. 40X.

Zone C Field

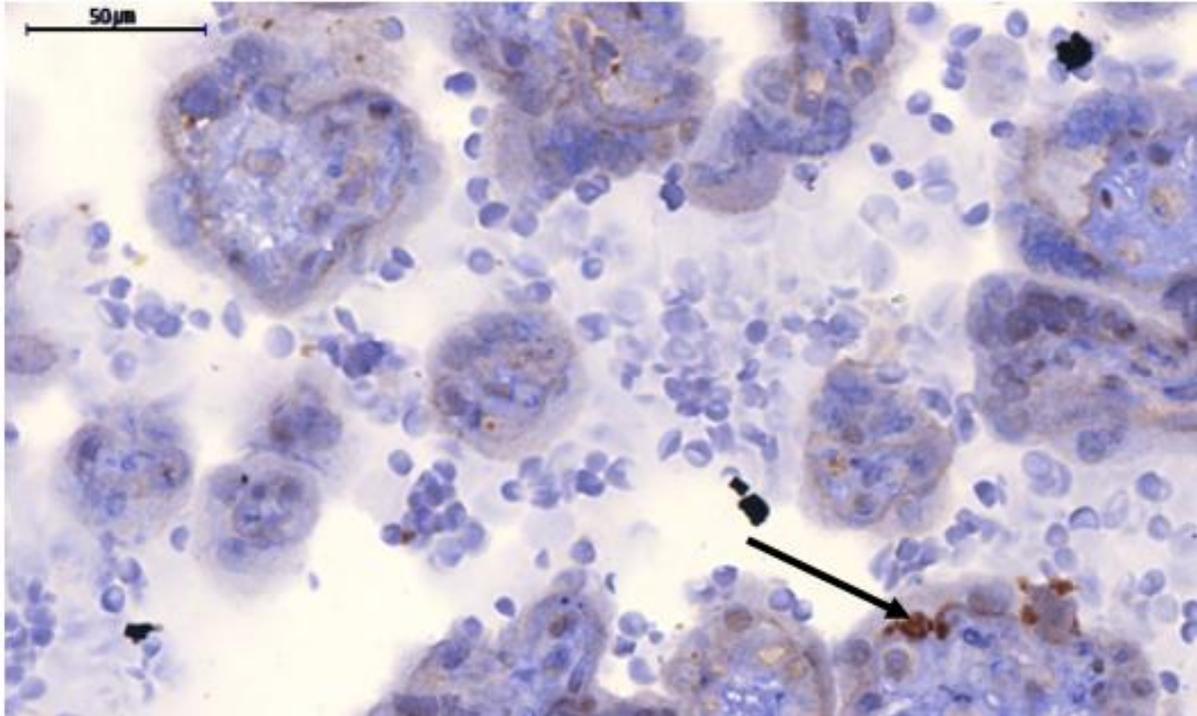


Figure 7: illustrate the M30 Cytodeath reaction (Black Arrow). 40X.

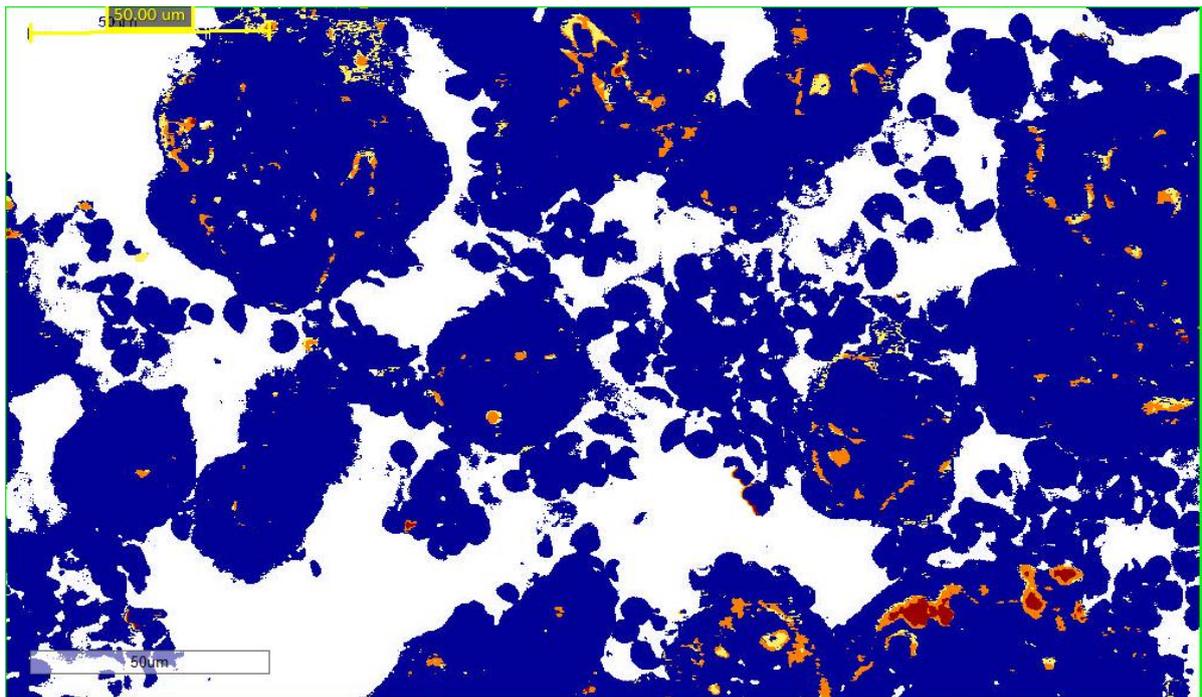


Figure 8: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

Zone D Field

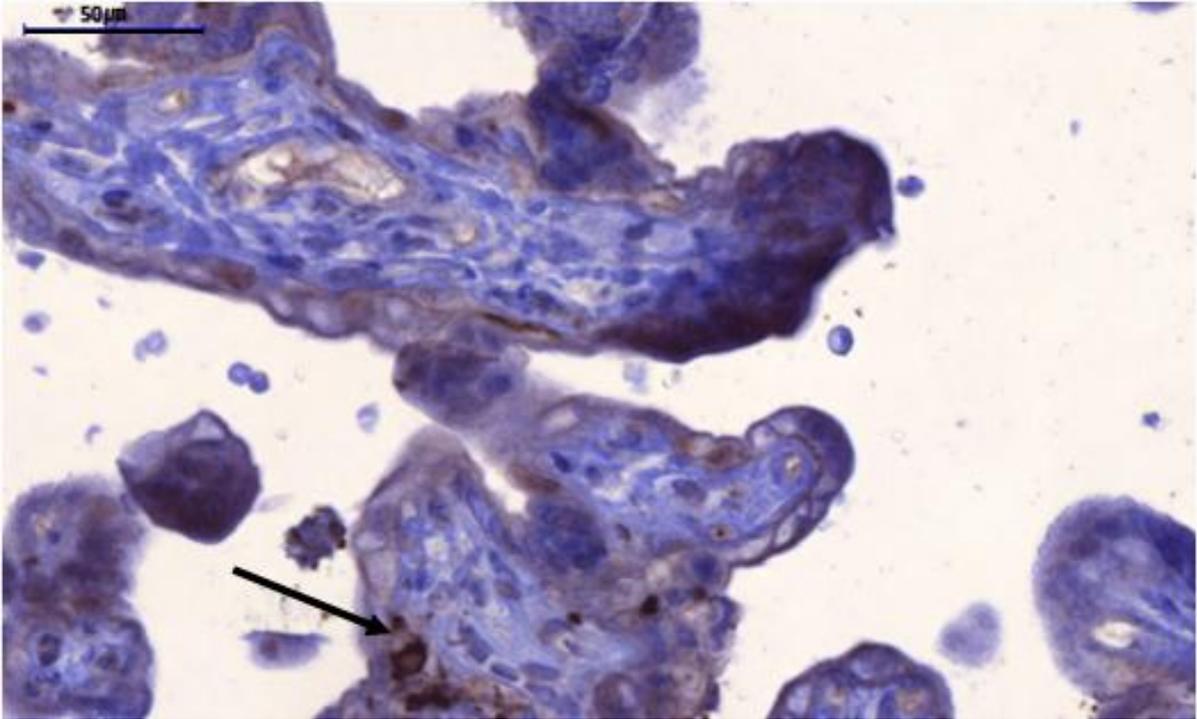


Figure 9: illustrate the M30 Cytodeath reaction (Black Arrow). 40X.

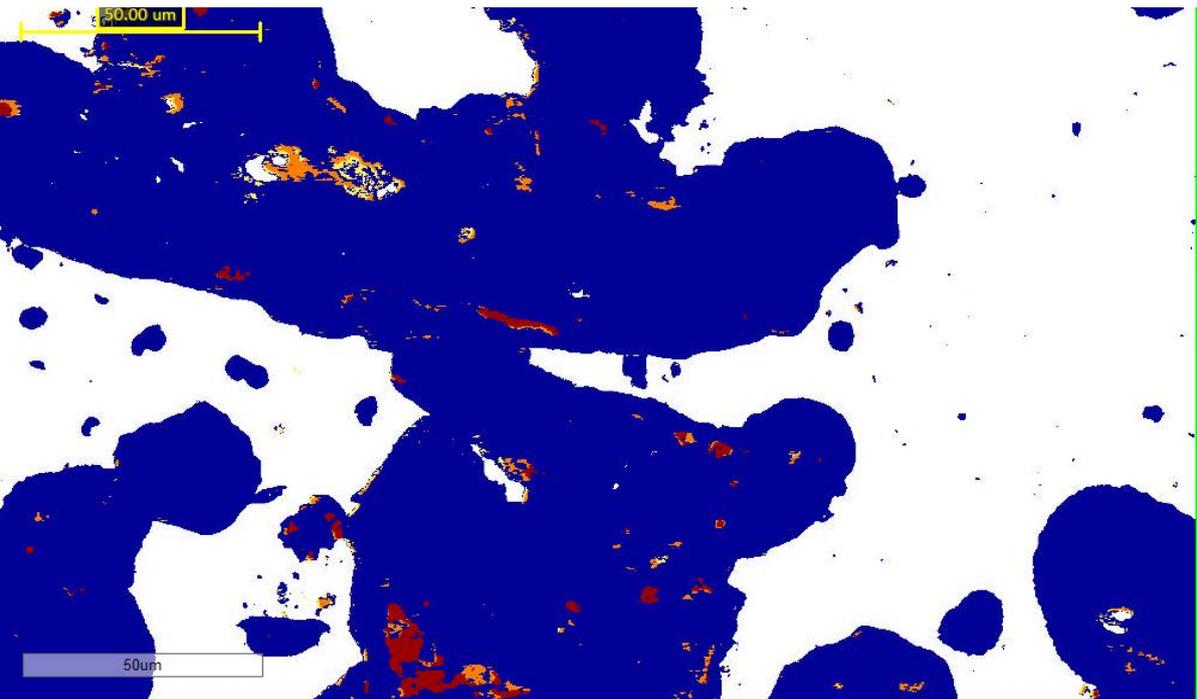


Figure 10: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

Zone A Field

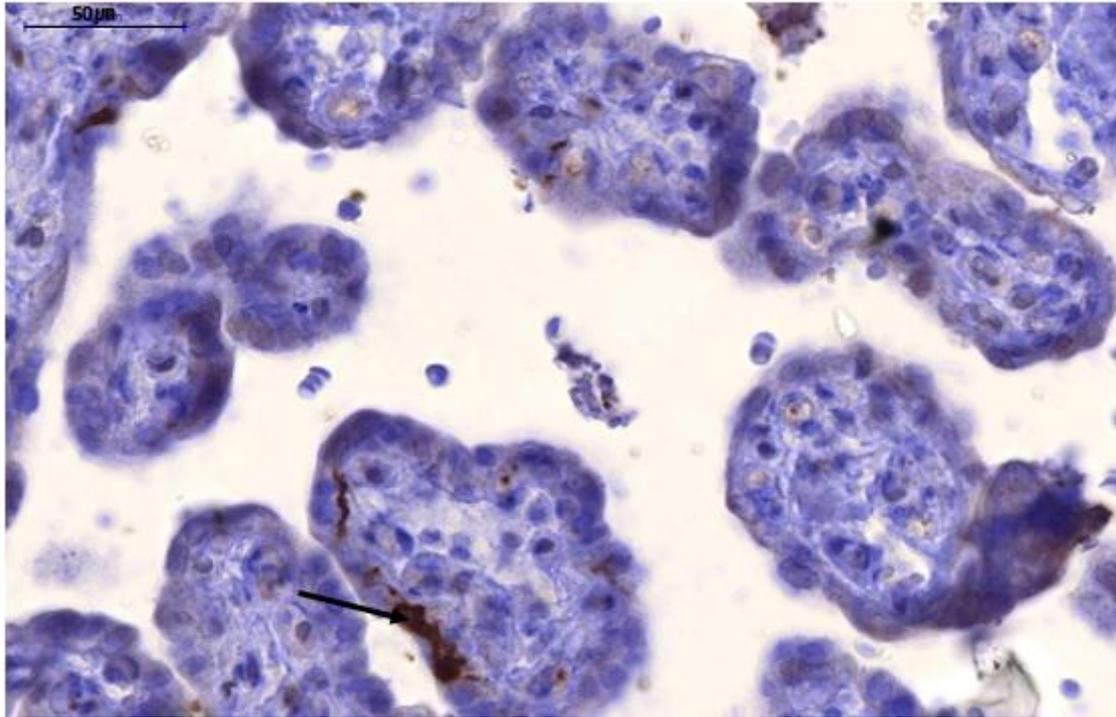


Figure 11: illustrate the M30 Cytodeathreaction (Black Arrow) 40X.

Zone B Field

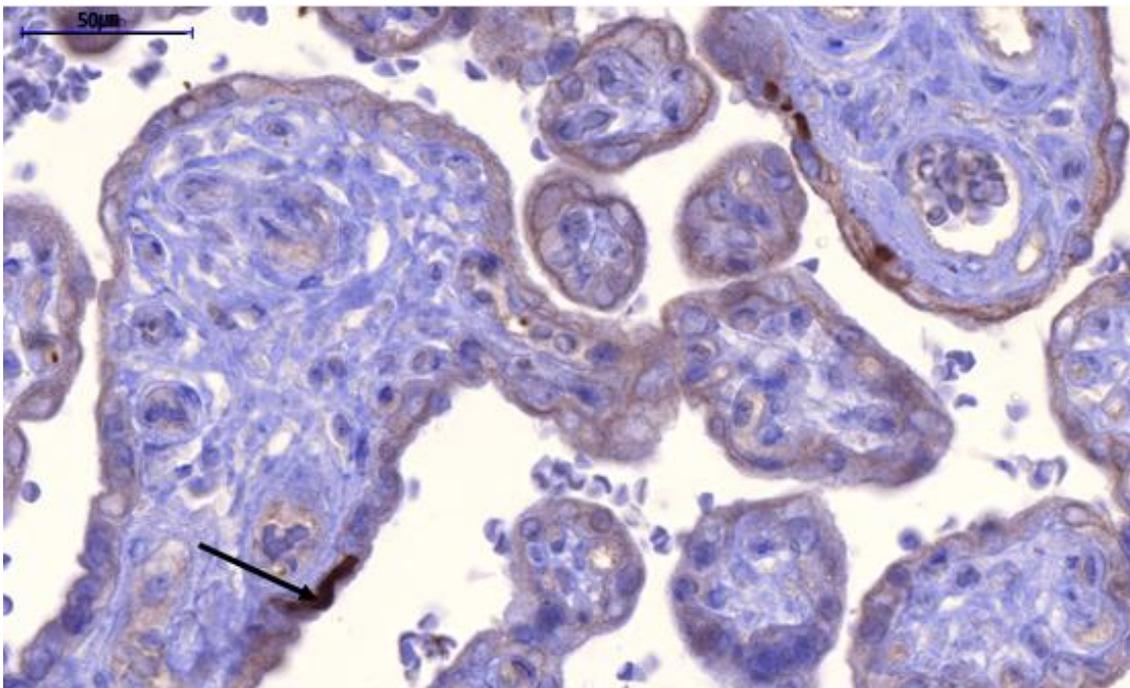


Figure 12: illustrate the M30 Cytodeath reaction (Black Arrow) 40X.

Zone C Field

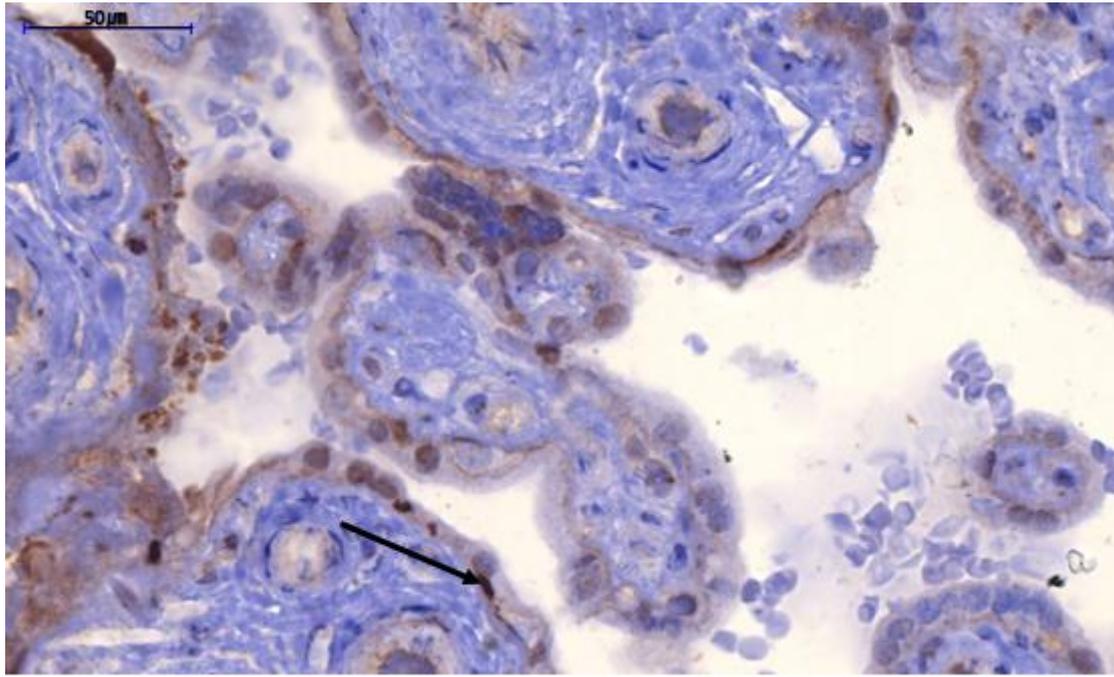


Figure 13: illustrate the M30 Cytodeath reaction (Black Arrow) 40X.

Zone D Field

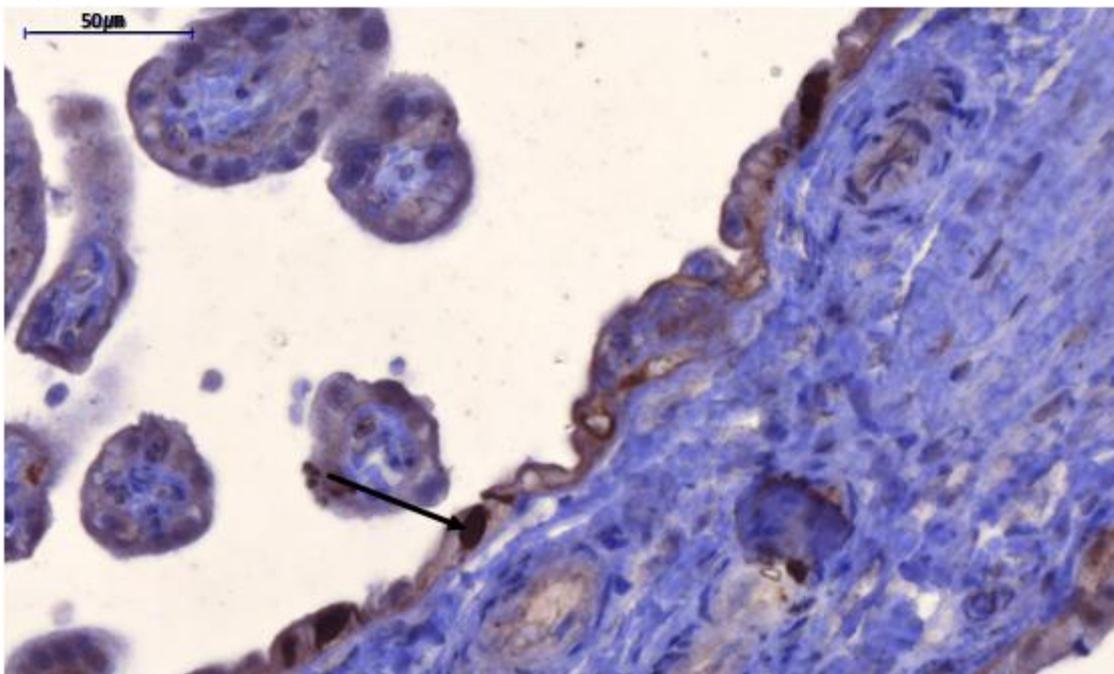


Figure 14: illustrate the M30 Cytodeath reaction (Black Arrow). 40X.

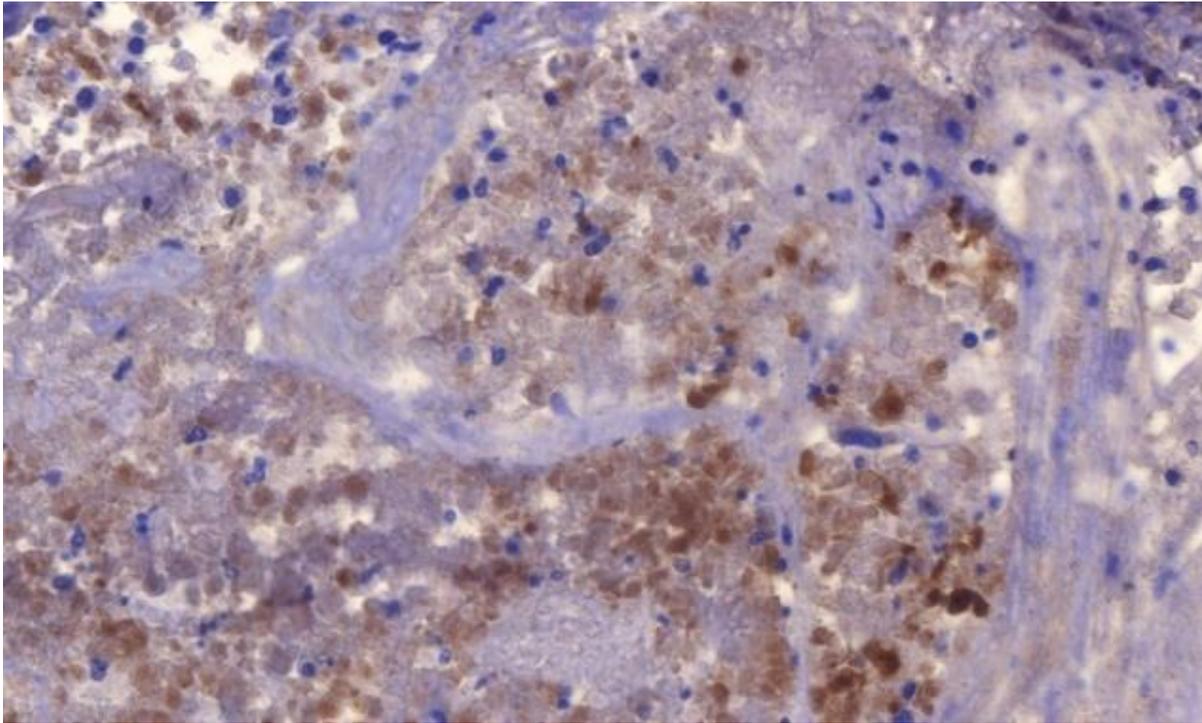


Figure 15: illustrate the positive control of adenocarcinoma of human colon.40X.

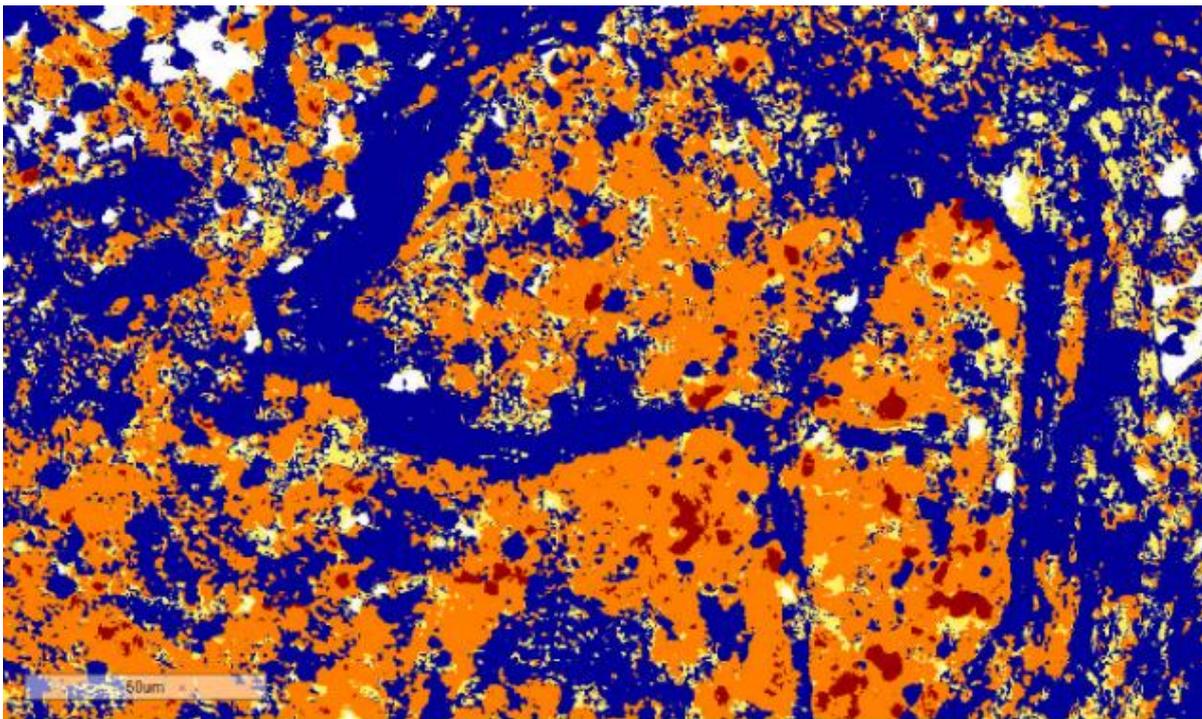


Figure 16: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

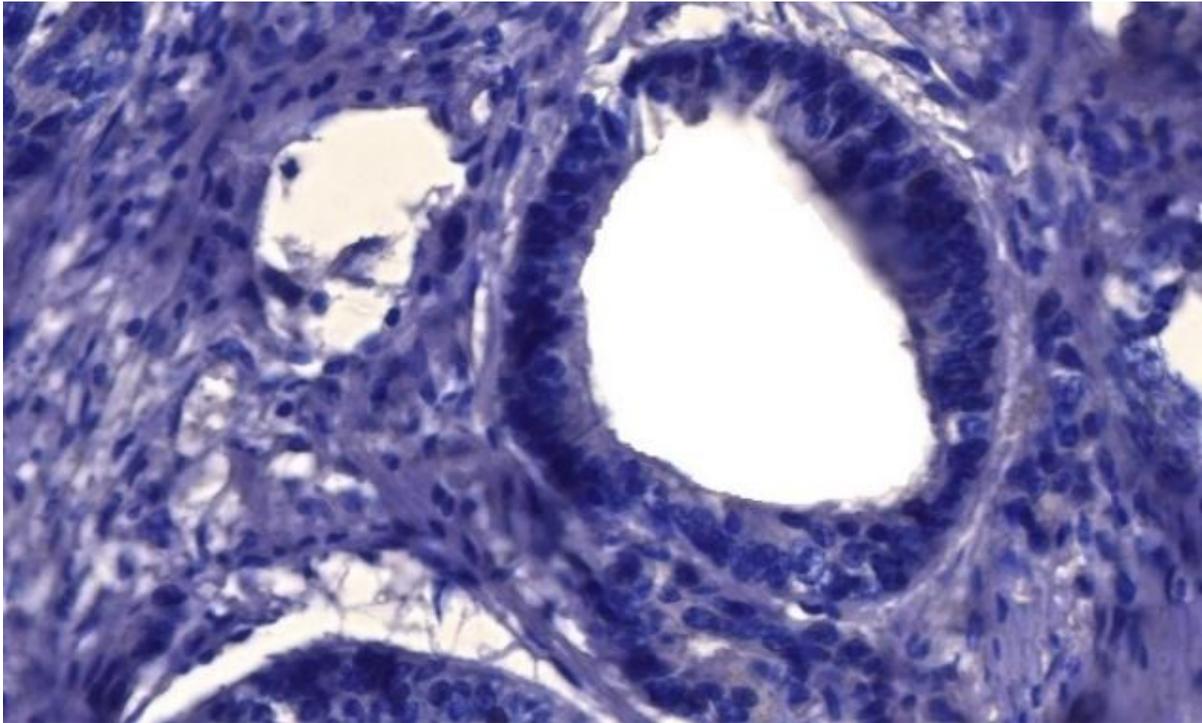


Figure 17: illustrate the negative control of adenocarcinoma of human colon 40X.

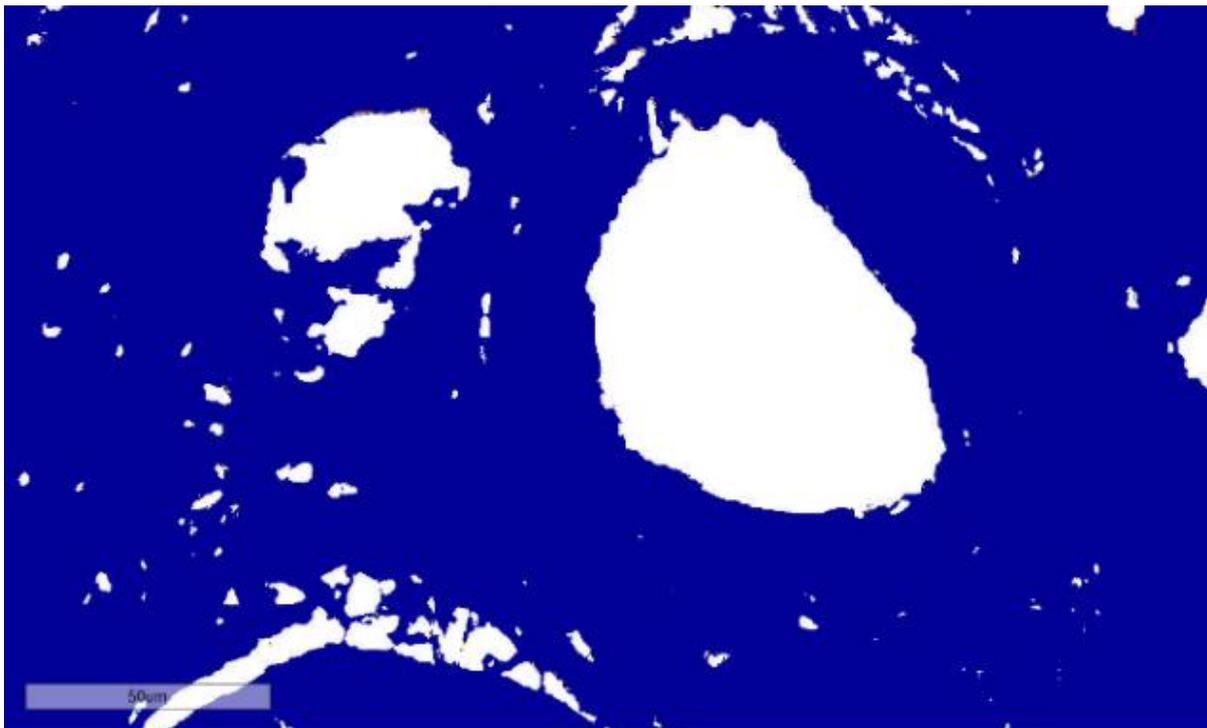


Figure 18: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

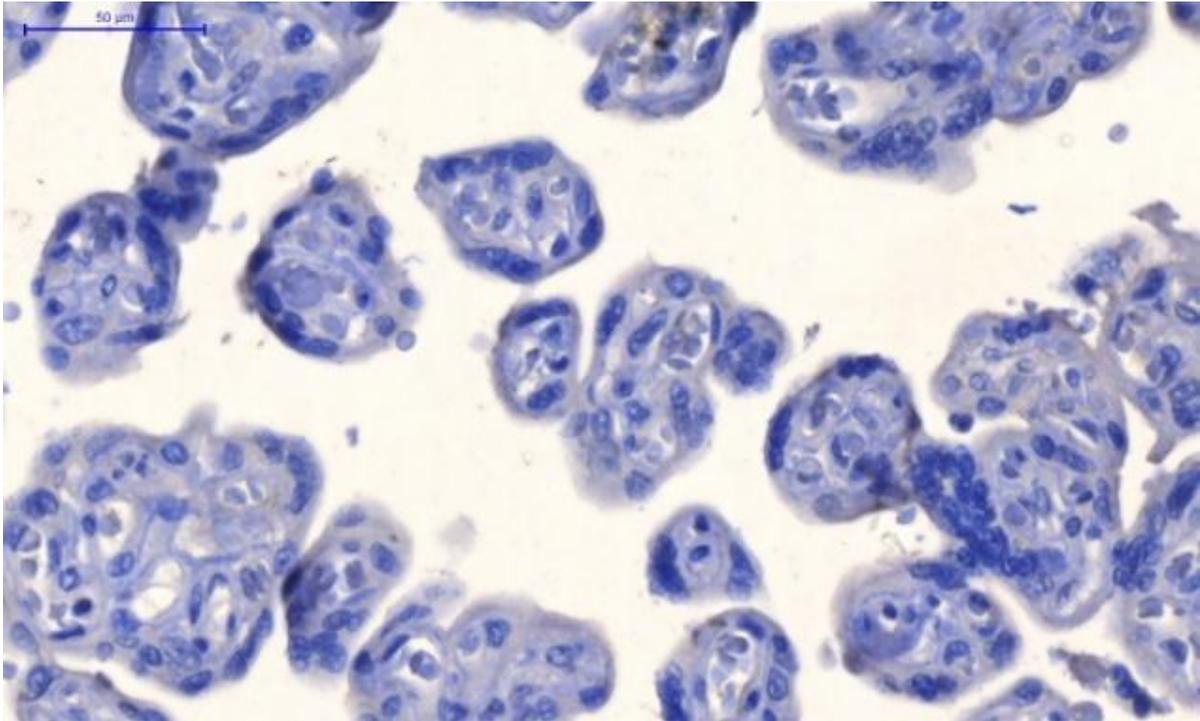


Figure 19: illustrate the negative control of human placenta.40X.

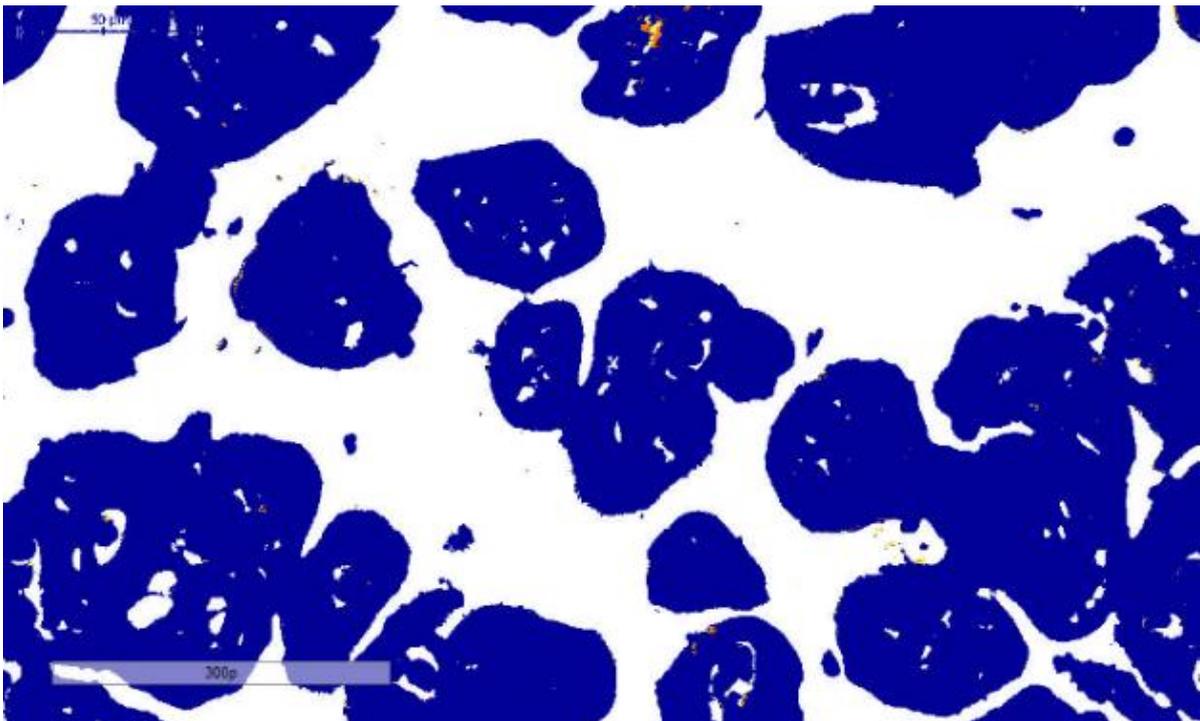


Figure 20: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

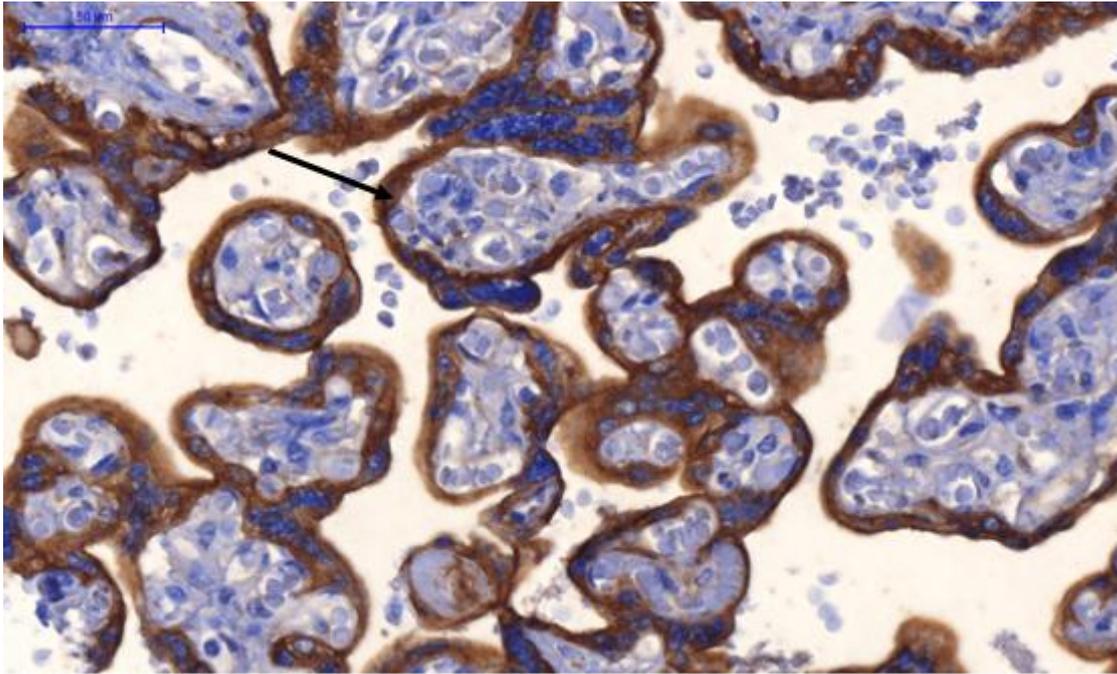


Figure 21: illustrate the CK 18 control of the cytoskeleton of human trophoblast placenta (Black Arrow).40X.

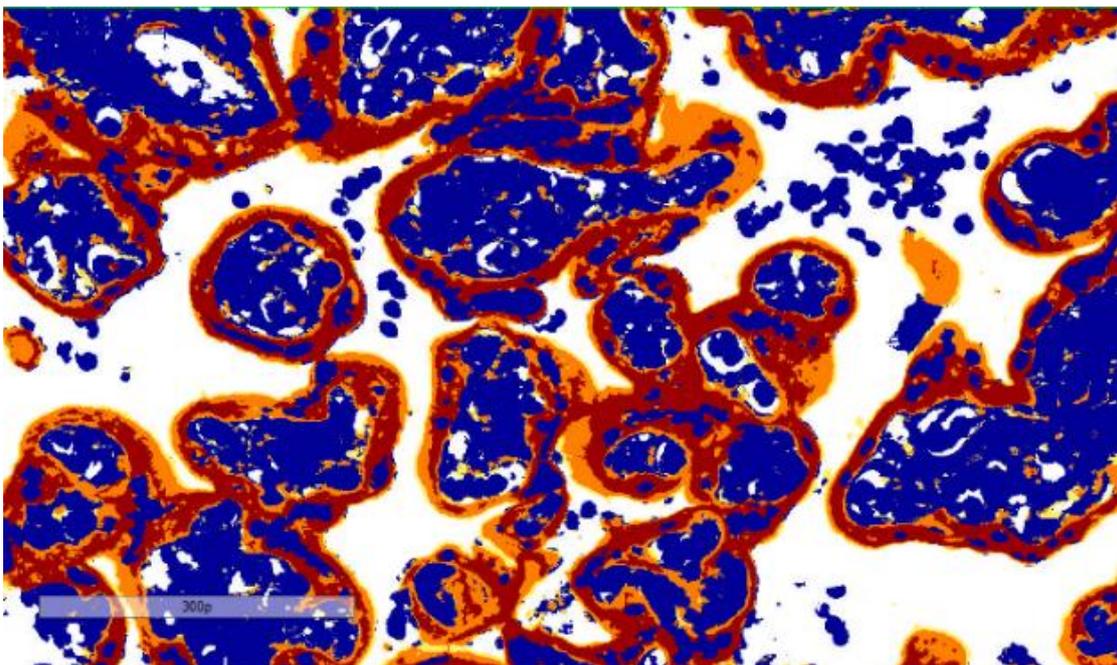


Figure 22: illustrate the color constricts resulted from Aperio image scope software analysis.40X.

Table 1: illustrate the outcomes value of M30 immunostaining in placental villi between A, B, C and D, using Anova test.

Groups	Mean Positivity ±Std. error	Sig.
A	3.9720±0.43964	0.188
B	2.7696±0.44111	
C	3.1647±0.46386	
D	2.7993±0.41830	

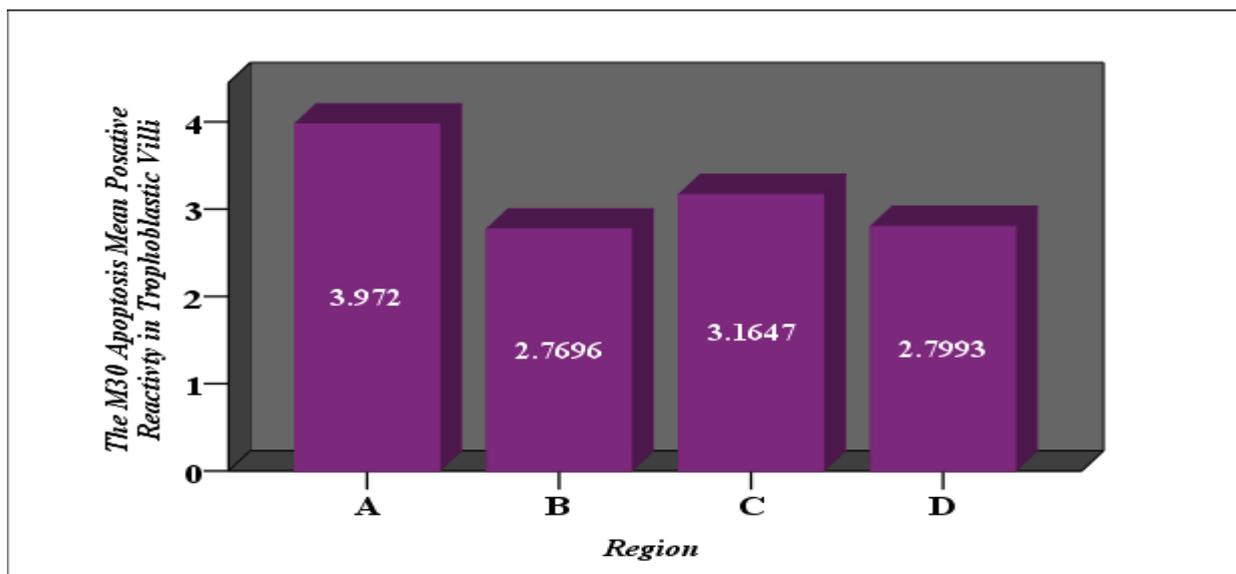


Figure 23: illustrate the mean number of M30 immunoreactivity in apoptotic trophoblastic villi.

Table 2: illustrate the outcomes value of M30 immunostaining in placental villi between zone A and zone B, using t- test.

Groups	Mean positivity ±Std. Deviation	t	Sig(2-tailed)
A	3.9720±2.94922	1.931	0.057
B	2.7696±2.95904		

Table 3: illustrate the outcomes value of M30 immunostaining in placental villi between zone A and zone C, using t- test.

Groups	Mean positivity ±Std. Deviation	t	Sig(2-tailed)
A	3.9720±2.94922	1.263	0.210
C	3.1647±3.11169		

Table 4: illustrate the outcomes value of M30 immunostaining in placental villi between zone A and zone D, using t- test.

Groups	Mean positivity ±Std. Deviation	t	Sig(2-tailed)
A	3.9720±2.94922	1.932	0.057
D	2.7993±2.80605		

DISCUSSION

The pattern of the trophoblastic villous turn over that was expressed by M30 Cytodeath (ROCHE) immunostaining

in the four zones (A, B, C, and D) obtained from maternal side of the largest diameter of the placenta were comparable and P value > 0.05. and the P value = 0.188, using Anova test.

The M30 CytoDeath immunoreactivity between Zones (A and B), (A and C), (A and D), was statistically non-significant and p value = (0.057, 0.210, and 0.057) respectively, using an independent samples t test. These results were comparable with Soni and his colleagues (2010), who find the apoptotic index for the human full-term placenta was (1.15 ± 0.21) as $(M \pm S.D.)$, they state that the apoptosis was acquired for normal pregnancy development in which the number of apoptotic trophoblast mean was higher at the first term placenta then decrease in the second term, and when it became full term the apoptosis rate was low. Moreover, Aban *et al.*, 2004 find in a study that the M30 index was (6 ± 5.55) as $(M \pm S.D.)$ in an Immunohistochemical evaluation when was carried out only in the epithelial component of the trophoblastic cells. Sharma *et al.*, 2012 discover in a study to assess the apoptosis in trophoblastic cells of placentas of normotensive non-protein-uric pregnant women; that the (apoptotic index) with M30 immunostaining in normal placenta was (61.9 ± 14.4) as $(M \pm S.D.)$ in trophoblast cells and (0) in stromal cells.

Jordan and Butchko 2002, reported that the level of epithelial cell apoptosis present within placenta obtained from normal pregnancies using M30 CytoDeath Monoclonal primary antibody was (1.57 ± 0.284) as $(M \pm S.E.)$. While Smith and his colleagues 1997 find in a study an apoptotic rate of 0.05% in normal term placenta, whereas Austgulen and his colleagues 2002 observed an apoptotic rate of approximately 0.03% in villous tissues. The somewhat lower apoptotic rate found in this study may partly be due to the strict criteria used. However, there were no differences in the apoptosis incidence in different parts of the same placenta. The apoptotic index in placental tissue from uncomplicated pregnancies was 0.93 ± 0.12 (Axt *et al.*, 1998).

In fact, apoptosis was mostly located in the trophoblast and stromal tissues. There were no significant differences in the incidence of apoptosis in different parts of the placental tissue. Meanwhile, apoptosis could be expressed in the human term and post-term placenta, with upgraded incidence in post-term placental tissue, suggested potential roles of apoptosis in the mechanism of parturition and the senescence of the placental (Axt *et al.*, 1999). In a study of Austgulen and his colleagues 2002, also demonstrated that the number of M30-positive cells in villous tissue fields varies in placentas; most M30-positive cells were syncytiotrophoblast. Approximately half of the positive cells were scattered cells within syncytiotrophoblast layer. Some of the syncytial knots also stained for M30. However, a few positively stained syncytial knots were found in the intervillous spaces. In areas with increased intervillous or perivillous fibrinoid, M30-positive cells were abundant. In fact, the total number of M30-positive cells accompanied with increasing intervillous or perivillous fibrinoid was approximately equal to that founded in the syncytiotrophoblast layer. The appearance and localization of positive cells in the areas with fibrinoid

suggested that they were apoptotic syncytiotrophoblast. However, these researchers demonstrated a very few M30-positive cells in the villous stroma.

In this study the non-significant mean of M30 immunostaining in placenta trophoblastic villi declined the hypothesis which stated that the eccentric umbilical cord insertion was eccentrically inserted into the placenta when the apoptosis mean immunoreactivity higher in zone A while the other zones (B, C, D) is lower with equal mean immunoreactivity values, hypothetically if the zone A have higher rate of M30 apoptosis mean immunoreactivity than the B, C, D, it means that the growth in this area will become less than the other area and that effect umbilical insertion (assumed the umbilical insertion is centrally) by sliding the umbilical cord from the center of the placenta towards the margin. This referred to that the apoptosis in the trophoblast had no relation to eccentric umbilical cord insertion and the programmed cell death is only to preserve the optimal functions activity of human placenta. The eccentric umbilical cord insertion may be correlated to another factor such as plane of implantation, differential placental development that derived from uterine conditions (Kaplan, 2007).

Variations in the site of insertion of umbilical cord are suggested to be resulted from the process known as trophotropism (Robinson *et al.*, 1983) in which the chorionic frondosum or the early placenta migrate with advances of gestation to ensure a good blood supply from the more richly vascularized areas (Monie 1965).

The location of the inserted umbilical cord affected the process of the development of the human placenta and the placental location at early gestation, suggested that this process might be affected by poor blood supply from the low uterine segment when umbilical cord insertion site is close to the internal cervical os (Hasegawa *et al.*, 2009).

The eccentric cord insertion has a little measurable relation with placental shape in observed or simulated placentas. However, placentas with a displaced cord revealed clear reduced transport efficiency, hence in a smaller birth weight for a given placental weight. Placentas with eccentric umbilical cord insertion have a sparser chorionic vascular distribution, as measured by the associated vascular distance. Even if typically, a placenta with a non-centrally insertion is of a normal round shape, its vasculature is less metabolically effective. These findings show another method by which altered placental component may affect the fetal environment, influencing birth weight and potentially contributing to later health risks (Yampolsky *et al.*, 2009).

Additionally, when the cytotrophoblast layer became thin and aged by time, it was necessary to this layer to be replaced and this was done by a process called apoptosis

or programmed cell death or cell turnover, which take place in the syncytium ending by syncytial knots formation. The M30 Mab recognizes a peptide generated after caspase directed cleavage of a specific epitope within the cytokeratin 18 molecule. M30 CytoDeath Mab does not react with trophoblast cells undergoing necrosis or apoptotic cells that do not contain cytokeratin 18, only those cytokeratin 18 positive cells undergoing caspase-directed apoptosis (Leers *et al.*, 1999).

The apoptosis began in the trophoblast when the proliferated cytotrophoblast activated by external trigger, this activation leads to activate the caspase 8. As soon as the caspase 8 was activated, the syncytial fusion began, in which the apoptotic cytotrophoblast start to fuse to the syncytium. During the development of human placenta, the cytotrophoblast differentiate and merge with the syncytiotrophoblast, allowing for growth of the syncytium and for re-epithelization of aged or damaged regions of villi. This process of trophoblast turnover is regulated to ensure normal fetal development (Li *et al.*, 2014). However, Sharp *et al.*, (2010) stated that this stems from two observations, first that caspase 8 is involved in the fusion of cytotrophoblast with syncytiotrophoblast, and second that some syncytiotrophoblast nuclei exhibit morphological features of apoptosis with peripheral chromatin condensation and gradual pyknosis.

The apoptosis in the normal CT is initiated by its fusion into the ST by activation of caspase 8, therefore an inactive form of procaspase3 would concomitantly be integrated into the ST, which will finally lead to the ST apoptosis. This has been supported by the studies of (Black *et al.*, 2004; Gauster and Huppertz, 2010).

M30 Mab recognizes a caspase 3 directed cleavage event within cytokeratin 18, a protein widely distributed in epithelial cells, of which trophoblast cells are classified (Jordan and Butchko, 2002). In fact, normal samples treated with anti-active Caspase 3 antibody showed positive reaction only in the ST (Bosco *et al.*, 2016) which mean that the cleaved cytokeratin18 must be present in the syncytium and detected by M30 Cytodeath. As it has been proposed, by some way yet to be determined by (Cao and Kaufman, 2014), (Szegezdi *et al.*, 2006) that normal ST apoptosis would be reactivated by activation of caspase 3 with expulsion of normal syncytial knots towards the hematic chamber. The human placental trophoblastic villous differentiation involves syncytial fusion of the cytotrophoblast to form syncytiotrophoblast. Early apoptosis cascade stages were described to be included in this differentiation process (Black *et al.*, 2004).

Previous publications on trophoblast apoptosis have presented controversial data not in agreement with recognized trophoblast biology: in 1998 Kokawa and his colleagues presented an incidence of apoptosis in villous cytotrophoblast ten times higher as compared to villous

syncytiotrophoblast whereas other authors described an extremely low incidence of villous cytotrophoblast apoptosis (Mayhew *et al.*, 1999).

For this project we decided to employ M30 to quantify trophoblast apoptosis since the other markers tested in a pilot series (active caspase 3, lamin B, PARP, and TUNEL) displayed numerous disadvantages. All of the latter markers are not specific for trophoblast and thus require either double staining or additional evaluation of anti-cytokeratin stained serial sections. Fixation-sensitive antigens such as the active form of caspase 3 and the intact forms of lamin B and PARP are likely to be altered in the archival material due to its processing and age (Kadyro *et al.*, 2003).

M30 immunohistochemistry is an attractive alternative to the TUNEL test for the application on placental tissues. (A) This antibody is specific for epithelial apoptosis events (in the placenta: trophoblast apoptosis) since it detects caspases mediated cytokeratin 18 cleavage products. Double staining for cytokeratins is not necessary. (B) The staining protocol is very simple and not sensitive to inter-individual variations. (C) The antigen is stable and not sensitive to fixation or variations in tissue processing. (D) Cytokeratin cleavage and thus M30 staining take place also when endonuclease activation during apoptosis is bypassed (Pampfer, 2000).

Necessaries and the need to find methods that accurately identify the apoptotic cells. One of the changes occurring in the cells undergoing apoptosis is the DNA fragmentation by endonuclease. This change occurs very late during apoptosis cascade for a very brief period of time (Collins *et al.*, 1997). Moreover, this DNA fragmentation also occurs in the necrotic cells (Grasl-Kraupp, *et al.*, 1995). Therefore, TUNEL assay may not be a sensitive and specific test for identifying only the apoptotic cells. Other characteristic feature of the epithelial cell apoptosis is the caspase-mediated cleavage of cytokeratins leading to disorganization of the cytoskeleton. Proteolysis of cytokeratins 18, 19 is involved in apoptosis degradation. These apoptosis-specific proteins have been identified as markers of increased cellular turnover (Hubert *et al.*, 2007), (Stefan *et al.*, 2009). Cytokeratin 18 has widespread distribution and is largely expressed in simple, non-stratified, ductal, and pseudostratified epithelia along with normal trophoblasts. Cytokeratin 18 when cleaved by caspase 3, 7 liberates a neoepitope, which can be identified by the M30 monoclonal antibody even before the disruption of membrane asymmetry and DNA strand break occurs (Caulin *et al.*, 1997). Thus, M30 immunostaining becomes an important tool for identifying apoptosis at very early stages.

Austgulen *et al.*, (2002) stated that, in fields of villous tissue, most M30-positive cells were CK18-positive syncytiotrophoblast. Since TUNEL detects apoptosis in

both epithelial and non-epithelial cells, more cells were positively stained with TUNEL than with M30 in some tissue fields. However, our observations suggest that M30 was more sensitive than TUNEL in recognizing apoptotic trophoblasts and had less nonspecific staining than TUNEL.

Cytokeratin 18 is a type I intermediate filament of epithelial cells, with a caspase-9 specific cleavage site at aspartate397 in the terminal of this protein, leading to the formation of a specific neopeptide recognized by the antibody M30. Cleavage of cytokeratin 18 is a very early stage in apoptotic signaling, preceding phosphatidylserine externalization (Ndozangue-Touriguine *et al.*, 2008).

Apoptosis is considered as a normal part of villous trophoblast turnover and syncytiotrophoblast formation from zytotrophoblast in the human placenta (Scifres and Nelson 2009). Meanwhile, Sharp and his colleagues (2010) concluded that the apoptosis is a feature of villous trophoblast throughout pregnancy and is an essential feature of placental invasion, cytotrophoblast fusion, and syncytiotrophoblast function as well as potentially playing a role in maternal immune tolerance. This process is not uncontrolled or haphazard in nature.

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