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The expression of Ki-67 marker in the hydatidiform mole

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Abstract

Background: The hydatiform mole is charcaterized by a pathological proliferation of the trophoblast. Its evaluation could predict the progression to gestational trophoblastic neoplasia. The aim of the study was the analysis of the proliferative activity of the villous trophoblast in the molar vs non-molar lesions.
Material and methods: p-57 and Ki-67 were evaluated by immunohistochemistry in 15 cases of hydatidiform mole and 18 cases of abortions.
Results: The hydatidiform mole was divided based on the immunoexpression of anti-p57 into: complete (8 cases) and partial type (7 cases). The distribution score of Ki67 immunoreactivity in the villous cytotrophoblast was: complete mole: +3 – 8 cases; partial mole: +1 – 1 case, +2 – 3 cases, +3 – 3 cases; abortions on social indications: +1 – 6 cases, +2 – 9 cases; +3 – 2 cases. The mean and standard deviations were: 2.88±0.354; 2.29±0.756 and 1.82±0.728, respectively. The following statistical correlations were determined: complete vs partial mole ($p=0.014$), complete mole vs abortion ($p<0.01$) and overall cases of mole vs abortion ($p=0.000034$).
Conclusions: Villous cytotrophoblast proliferative activity is high in the complete hydatidiform mole, and the immunoreactivity distribution index is highly positive and statistically true in the molar versus non-molar group. Immunohistochemical evaluation of Ki-67 in molar pathology is useful in differential diagnosis as a complementary method.
Key words: hydatidiform mole, Ki-67, trophoblastic proliferation.

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Introduction

The hydatidiform mole, as a component of gestational trophoblastic disease, is categorized into two subtypes: complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM). The maternal genetic component is present in the case of the partial form and absent in the case of the complete form [1]. CHM is a non-invasive placental disease characterized by hydropic, cystic deformations of the chorionic villi accompanied by pronounced trophoblastic proliferation. PHM comprises mainly two populations of chorionic villi: corresponding to the gestational age and villi with hydropic changes in the presence of expressed focal proliferative character [2, 3].

Complete molar, partial molar, as well as non-molar lesions in the germinal compartment are established during histomorphological examination and are frequently treated as inconclusive due to interobserver and intraobserver diagnostic variability. This lack of clarity in the diagnosis of early-term disturbed pregnancies with molar and non-molar profile is the result of the masking of morphological peculiarities in the choriovillary compartment [4, 5]. The histomorphological lesions are quite non-specific and do not facilitate the differential diagnosis in molar pathology, especially in the early period of evolution [6], considering also the association of hydropic and/or cystic stromal lesions [7].

The assessment of the risk of the development of persistent gestational trophoblastic disease and its subsequent management are affected by the variability of the diagnosis of histopathological lesions in early-term disturbed pregnancies, including molar pathology. Therefore the assessment of the trophoblastic epithelial proliferative profile has a priority importance in the diagnostic management. Thus, the elucidation of a marker for the differential diagnosis, together with the evaluation of the proliferative pattern, remains a subject for studies. A number of studies have shown the impact of paternally imprinted p57 gene expression in the differential diagnosis of molar vs non-molar pathology with incomplete differentiation [8, 9].

Ki-67 is a non-histone nuclear protein present in all active phases of the cell cycle, being virtually absent in quiescent G_0 cells. Ki-67 protein expression is strictly associated with cell proliferation, therefore it can be applied as a marker for dividing cells [10]. Therefore, the aim of the

study was to evaluate the proliferative activity of the villous trophoblast in the hydatidiform mole.

Material and methods

General characteristic. The study material was represented by the tissue samples obtained after medical abortion from 15 patients with short-term pregnancies (3-12 weeks) from the level III Perinatal Center, Mother and Child Institute, during 2019–2021, with the morphopathological diagnosis of hydatidiform mole. They were included in the L_I study group. The age of the patients was between 17-47 years with an average of 28.4±9.36 years. All the patients were examined by USG, in 5 cases morphological ultrasound aspects characteristic of molar hydatidiform structures were established.

The control material included tissue samples of the fetal conceptus taken after abortion on social indications/desire from 18 patients who constituted the control group (L_{II}). The age of the patients in this group was between 22-40 years with an average of 30.5±5.6 years. Clinical data were obtained from each patient's medical records. Specimens of liver were used for external negative control. The current research is the part of a larger study of disrupted early-term pregnancies.

The histological examination included the histoprocessing of tissue samples, the application of the usual histological method (hematoxylin-eosin) and the immunohistochemical method (anti-Ki67 and anti-p57) with the evaluation of microscopic features, as well as statistical processing.

Primary processing. The tissue material of the product of conception was collected at short term in obstetrics department with rapid fixation in 10% formalin solution, pH 7.2-7.4 to reduce the risk of early lysis of tissue material and superimposition of bacterial flora. The period of fixation in formalin solution was not more than 24 hours. The paraffin embedding system was DP500/CIT2002 (Bio-Optica, Italy). Histochemical and histological processing of the samples was performed on the histoprocessor „TISSUE-TEK, VIP 6AI" (Sakura, Japan), sectioning on the microtome HM325 (Thermoscientific) (USA). Sections with a thickness of 5 μm were spread on positively charged slides (APTACA, Italy).

Histological method. Sections were stained by the classical conventional hematoxylin-eosin (HE) method, using Mayer's hematoxylin (HEMM-36/21, BIOGNOST, Slovenia) and eosin Y 1% (EOY10-35/21, BIOGNOST, Slovenia). Sections for H&E were automatically stained with autostainer AUS-240, (Bio-Optica, Italy) and automatically mounted (TISSUE-TEK, ClasTM, Sakura, Japan). Appropriate sections (sufficient tissue material) were selected for immunohistochemical staining.

Immunohistochemical method. The immunohistochemical assays were performed by manually adapted operational procedures for two antibodies: anti-Ki67 (clone MIB-1, ready-to-use, FLEX, monoclonal mouse anti-human, Dako, IS626) and anti-p57 (clone 25B2, Novocastra Liquid Mouse Monoclonal Antibody for human p57 protein (Product code: NCL-L-p57: Leica Biosystems Newcastle Ltd, Newcastle, UK) with the application of 2 detection systems: EnVision™FLEX, high pH (K8000) [11] and Novolink™MaxPolimer, Leica (RE7280-K) [12]. The conventional immunohistochemical method was applied (Table 1). Deparaffinization was performed in two toluene baths (code UN1294, Sigma-Aldrich) of 5 minutes each, a mixed toluene and 99.9% alcohol bath (code 06-10077F) for 5 minutes, followed by 2 baths in 99.9% alcohol with rehydration in distilled water in 2 intakes of 10 minutes each.

Application of primary antibodies was preceded by exposure of tissue sections to Target Retrieval Solution, Flex, high pH (50x) (code DM828, Dako) for anti-Ki67 antibody and Na citrate solution, pH 6.0 for anti-p57. The exposure took place in a water bath at a temperature of 95°C-96°C of the unmasking solution, for 20 minutes, with a total pretreatment and posttreatment time of 60 minutes. Sections were incubated with primary antibodies for 30 minutes at room temperature. Endogenous peroxidase blocking was performed by applying Peroxidase-Blocking Solution for 5 minutes, and DAB (3,3'-diaminobenzidine) was applied as a chromogenic substrate for 5 minutes. Nuclei were counterstained with Mayer's hematoxylin (HEMM-36/21, BIOGNOST, Slovenia) when using the EnVision™FLEX detection system, high pH. In the case of the Novolink™MaxPolimer system, Leica hematoxylin (RE7164) was applied. The final product of the reaction was expressed by staining the nuclei in brown. Then, the panel of histological slides was subjected to the procedure of dehydration and clarification in 2 batches of absolute alcohol, one batch of mixed alcohol and toluene, and three batches of toluene, each exposure being 5 minutes. The final procedure consisted of mounting the slides with BMC-100 solution. In the manual immunohistochemical staining procedure, the Sequenza™ Immunostaining Center was applied using the Thermo Shandon Coverplate.

Microscopic evaluation. Ki67 (nuclear proliferation receptor) protein expression was analyzed in the chorionic villus compartment (trophoblastic epithelial component). The regions with the highest cell density and nuclear expression (hot-spots) were identified at x100 magnification.

Table 1. Antibodies used: source, dilution, unmasking system, detection system, incubation time

Antibody/ clone	Sourse/ incubation time/ dilution	Retrieval system/ time	Detection/ time
p57/ 25B2	Leica Biosystems Newcastle Ltd, Newcastle, UK/ 30 min/ 1:100	Na citrate solution, pH 6.0/ Water bath at a temperature of 95°C-96°C/ 20 min	Novolink™Max Polimer, Leica/ 20 min
Ki67/ MIB-1	Dako/ 30 min/ ready to use	Dako Target Retrieval Solution, high pH/ Water bath at a temperature of 95°C-96°C/ 20 min	EnVision™-FLEX, High pH/ 20 min

Subsequently, was counted the number of immunopositive cells per 100 cells from 3 fields of view at x400 magnification. Then was determined the value of the Ki-67 proliferation index (PI/ %), that represented the percentage of immunopositive cells vs the total number of cells. The following score was assigned: 0 – absent, weakly positive (+1 in <10% of cells), moderately positive (+2 in 10-50% of cells) and strongly positive (+3 in >50% of cells).

Positive expression of p57 in the choriovillary and gestational deciduo-endometrial germinal compartment was determined based on nuclear positivity in decidual cells, villous trophoblast and intermediate extravillous trophoblast (positive internal control). p57 immunopositivity was interpreted as satisfactory (negative) when choriovillary stroma and chorionic villus trophoblast cells were completely negative or showed nuclear immunoexpression in less than 10% of cells, in the concomitant presence of the positive internal control. Chorionic villi and decidual plaques from normal human placenta served as positive external controls. The negative external control was represented by the hepatocytes (negative immunoreaction), being included in each research group. In all research sites cytoplasmic expression was considered nonspecific. The quantification method was applied according to Gupta M. et al. [13]. Quantification of positive cells was performed with an Axio Imager A2 microscope (Carl Zeiss, Germany) equipped with an AXIOCam MRc5 recording camera.

Statistical methods. The study results were stored and grouped in the MS Excel 2010 database. Data analysis was performed using the SPSS program (SPSS Statistics 23.0; IBM, Chicago, IL, USA). Descriptive statistics were applied with the determination of the arithmetic mean (M) of the Ki67 values and the standard deviation (SD). Were also compared the values of Ki-67 PI in the L_I vs L_{II}, as well as inside the L_I (complete vs partial hydatiform mole) by applying the t test. The results were considered statistically significant at a $p<0.05$.

Results

The evaluation of the cases included in the L_I study group showed that the definite diagnosis of hydatidiform mole was established in 33.3% of cases. In 66.7% of cases, due to the predominance of hemorrhagic syndrome, the clinical diagnosis was of endometrial glandular hyperplasia, ongoing pregnancy with spontaneous abortion or stagnant pregnancy.

Morphological examinations by usual methods of the abortive product in the early period in both groups determined 2 morphological types of fetal conceptus: type V – molar hydatidiform fetal conceptus (HMFC) and type VI – disorganized fetal conceptus (DFC) in various macroscopic variations of the material. In the material classified as type VI fetal concept, in 5 cases the predominantly mushy character was macroscopically attested (figure 1a-b).

Following the application of the anti-p57 antibody, according to the particularities of the immunomorphological profile attested at the site of the villous trophoblast (negative or positive immunoreaction from the villous cytotrophoblast), two subtypes of hydatidiform mole were deciphered: complete hydatidiform mole – 7 cases (46.7%) and partial hydatidiform mole – 8 cases (53.3%) (fig. 2). In all cases studied, the extravillous trophoblast was analyzed for the internal positive control, together with the intense expression in the decidual cells (fig. 3a). The hepatocytes that did not present a positive immunoreaction served as an external negative control (fig. 3b).

Later, the cases were analyzed in terms of the proliferative character of the villous trophoblastic epithelium, expressed by the Ki67 proliferation index (PI). The numerical values and their distribution in relation to the hydatidiform mole subtypes and the control group are elucidated in table 1.

According to the results, the maximum immunoexpression of the anti-Ki67 reaction at the level of the villous cytotrophoblast was attested in the group with molar pathology, the values of the complete molar subtype being

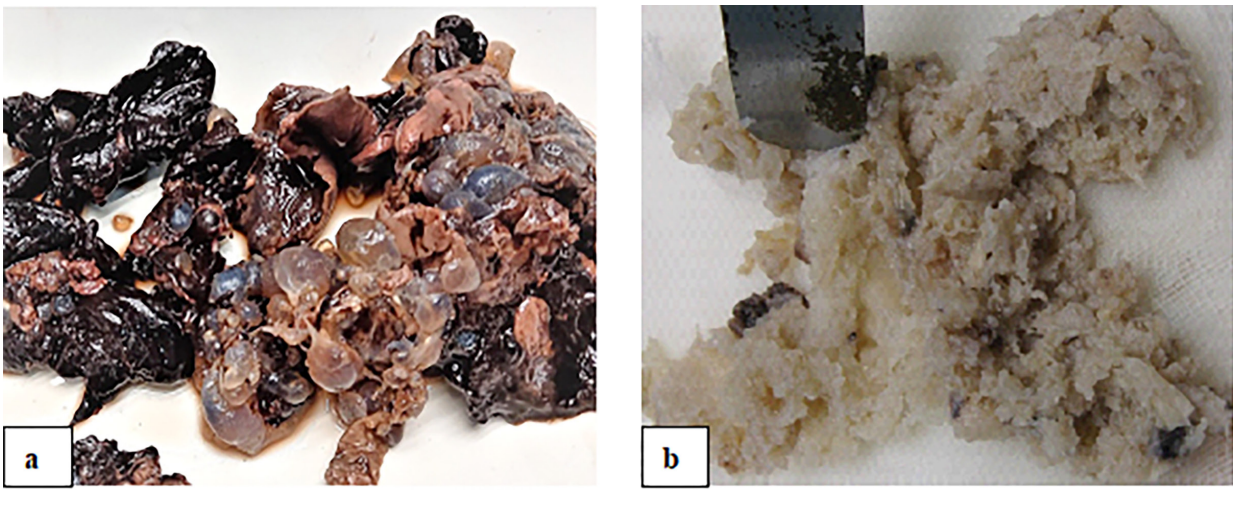

Fig. 1. Macroscopic features of the fetal concept: a) Molar aspect of the germinal sac in abortion with brown and hemorrhagic changes of the decidual plaques (HMFC); b) The mushy aspect of the disintegrated conceptus in medical abortion (DFC)

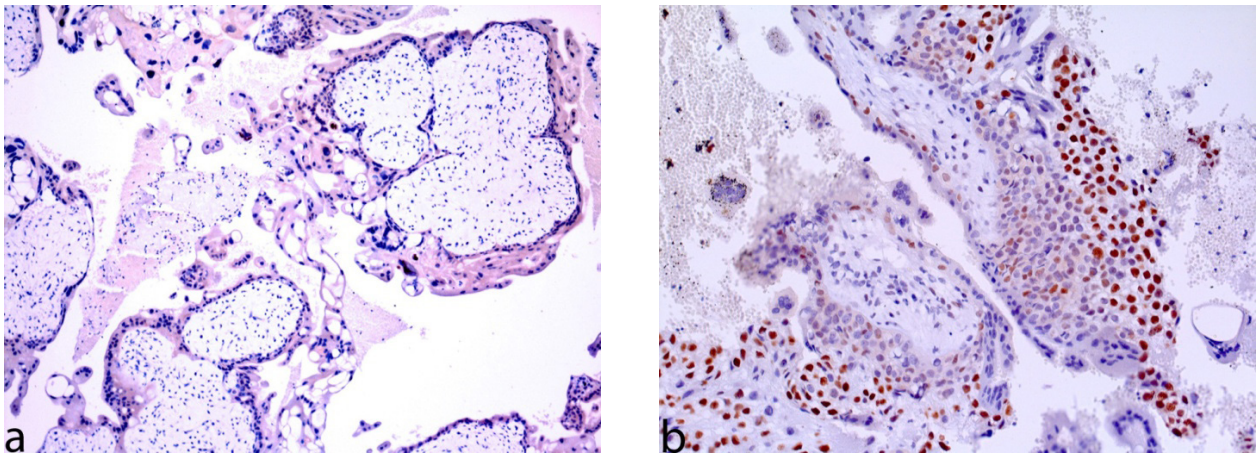

Fig. 2. Hydatidiform mole. a) CHM: Negative immunoexpression of villous trophoblast; b) PHM: Positive immunoexpression of villous trophoblast. Immunoreaction for anti-p57, DAB, × 100, 200

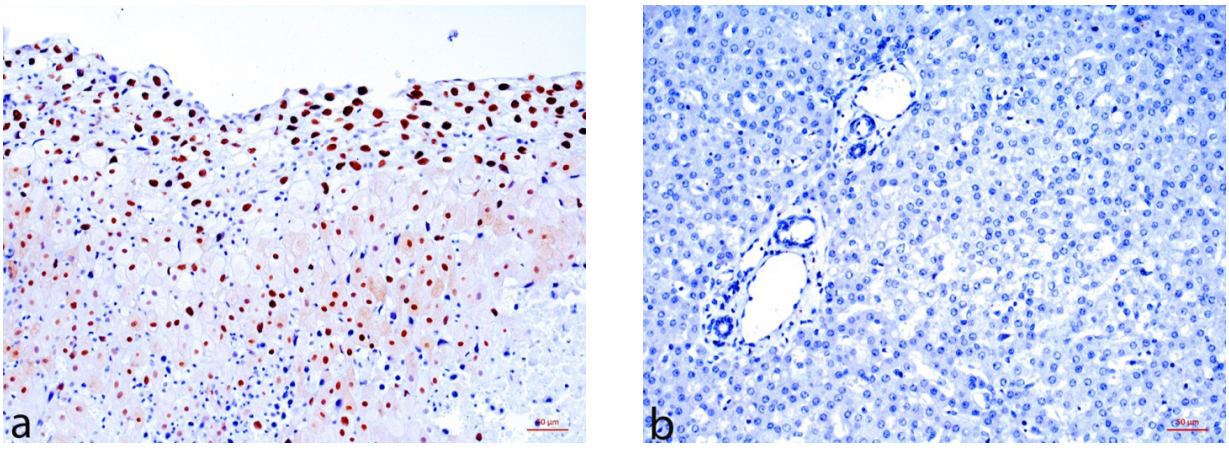

Fig. 3. a) Intense positive immunoexpression of p57 in decidual cells and extravillous trophoblast (positive internal control); b) negative imunoexpression in hepatocytes (positive external control). Immunoreaction for anti-p57, DAB, × 200

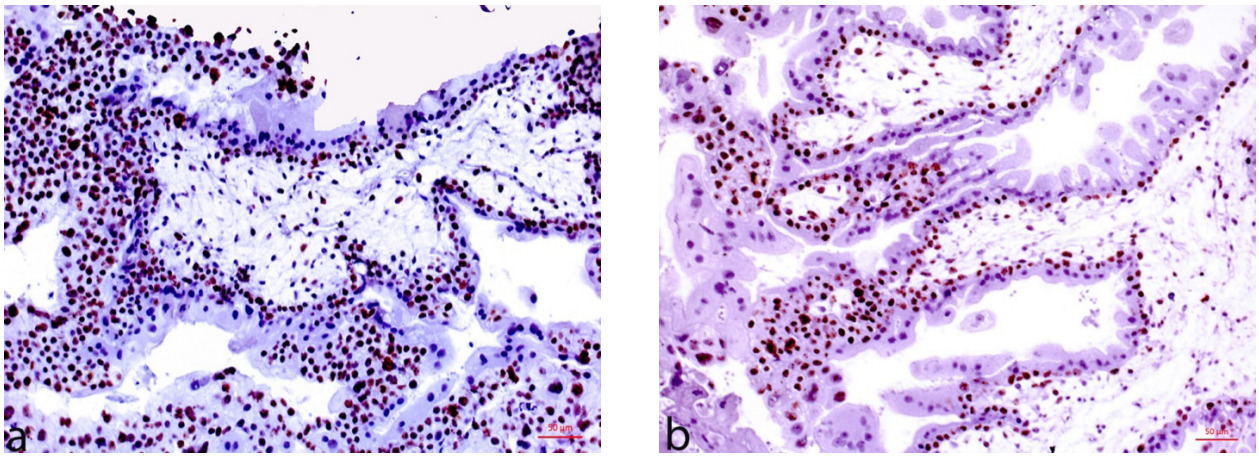

Fig. 4. CHM (a, b). Positive immunoexpression in the villous trophoblast. Mitotic activity with pronounced cytotrophoblastic proliferation. Distribution score +3. Immunoreaction for anti-Ki67, DAB, × 200

clearly superior both inside the group and also in relation to the abortion group. Immunoreactivity in the syncytiotrophoblast was not attested. The distribution of Ki67 immunoreactivity in the studied groups is reflected in table 2.

In the CHM group, the score was a maximum of +3 in 100% of cases (fig. 4). In PHM, equal values of +3 and +2 scores were attested (42.9% each) and only one case (14.2%) of a weak positive (+) immunoexpression (fig. 5). In the overall molar pathologies, the maximum score of +3

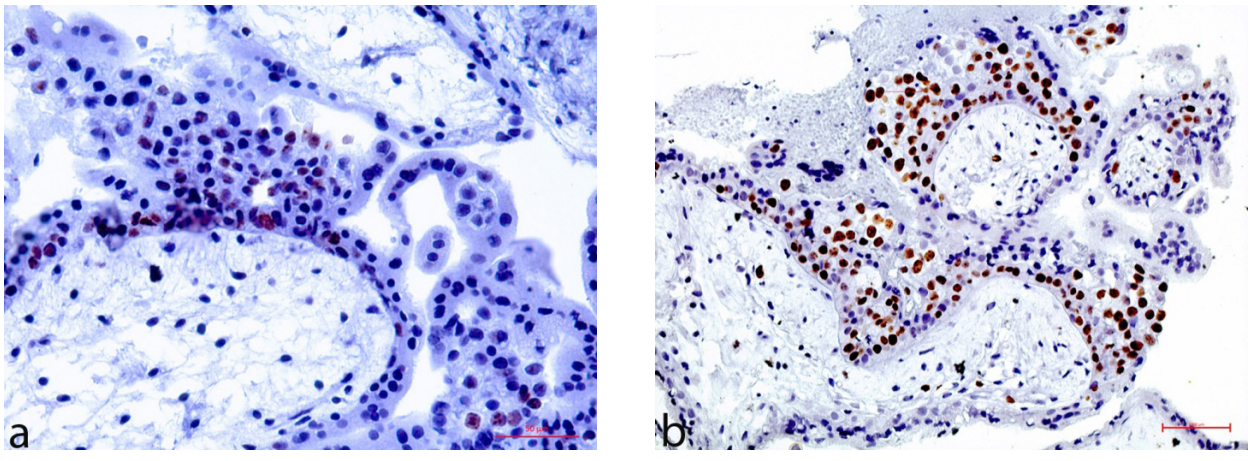

Fig. 5. PHM (a, b). Positive immunoexpression in the villous trophoblast. Distribution score: a) +2; b) +3. Focal proliferation. Immunoreaction for anti-Ki67, DAB, × 400, 200

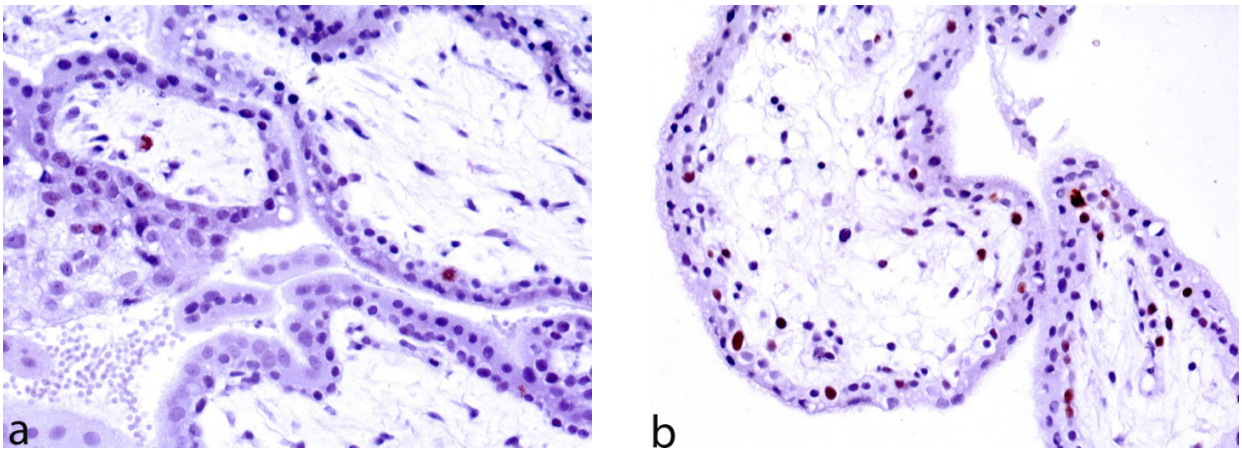

Fig. 6. Abortion on social indications/ desire (a, b). Positive immunoexpression in the villous trophoblast. Distribution score: a) +1; b) +2. Focal proliferation. Immunoreaction for anti-Ki67, DAB, × 400

Table 1. Mean value and standard deviation (M±SD) of the Ki67 marker in relation to the study group

Study group	Ki67 (PI)	
	Villous cyto-trophoblast	Villous syncytio-trophoblast
	M±SD	M±SD
CHM	87.25±4.59	0±00
PHM	48.71±29.66	0±00
HM (total)	69.26±27.99	0±00
AS/D	25.05±20.84	0±00

Table 2. Distribution of Ki67 immunoreactivity (%)

Study group	Cytotrophoblast/ score				Syncytiotrophoblast / score			
	0	+	++	+++	0	+	++	+++
CHM	-	-	-	8	-	-	-	-
PHM	-	1	3	3	-	-	-	-
HM (total)	-	1	3	11	-	-	-	-
AS/D	-	6	9	2	-	-	-	-

Note: „0" – absent immunoexpression; „+" weakly positive (<10%), „++" moderately positive (10-50%), „+++" strongly positive (>50%)

was attested in 11 cases, +2 in 3 cases and +1 in one case. When evaluating the distribution of immunoreactivity in the control group, the +2 score (9 cases/52.9%) was dominant, followed by the +1 score (6 cases/35.3%) and the +3 score (2 cases/11.7%) (fig. 6). The mean and standard deviations were: 2.88±0.354; 2.29±0.756 and 1.82±0.728, respectively

At the same time, when analyzing the obtained results, a coincidence of Ki67 immunoreactivity was observed in the following groups: CHM/ PHM/ AS/D in case of +3 score, PHM/ AS/D in case of +2 score and PHM/ AS/D in case of +1 score.

The mean values of Ki67 PI in each study group can be found in the table 3.

To test the hypophysis that each morphological entity was associated with statistically significant different mean values of Ki67 PI, a t-test was performed. The following statistical correlations were found: MHC vs MHP ($t_{15,71}$=3,402, p=0,014), MHC vs AS/D ($t_{13,16}$=11,71, p<0,01) and mole vs AS/D ($t_{1,64}$=5,105, p=0,000034).

Table 3. The mean values of Ki67 PI

	MD	N	Mean	Std. Deviation
Ki67citovPI	CHM	8	87.2500	4.59036
	PHM	7	48.7143	29.66319
Ki67citovPI	CHM	8	87.2500	4.59036
	AS/D	17	25.0588	20.84907
Ki67citovPI	PHM	7	48.7143	29.66319
	AS/D	17	25.0588	20.84907
Ki67citovPI	mole	15	69.2667	27.99354
	AS/D	17	25.0588	20.84907

Note: citov – cytovillous, PI – proliferative index, MD – morphological diagnosis, N – number of cases, CHM – complete hydatiform mole, PHM – partial hydatiform mole, mole – overall cases of hydatiform mole (CHM+PHM), AS/D – abortion on social indications/desire.

Discussion

Hydatidiform mole (HM) represents a heterogeneous group of lesions characterized by trophoblastic proliferative and hydropic-cystic choriovillary abnormalities in the germinal compartment, caused by abnormal fertilization. The differentiation of the two forms (subtypes) of HM is possible by cytogenetic analysis and based on ploidy. The 46XX karyotype, less often 46XY and very rarely the tetraploid for CHM were appreciated. In the case of PHM, a triploid genome (69XXX or 69XYY karyotype) or tetraploid consisting of a maternal and paternal haploid genome were determined, the latter reduplicated as a result of bisperm fertilization [14, 15]. This differentiation is of major importance in clinical management, a fact determined by the increased risk of persistence of trophoblastic disease or evolution into choriocarcinoma [16], patients presenting CHM being more frequently affected [17].

For the purpose of distinguishing the molar profile, the literature mentions the application of differential immunoreactivity of p57kip2 [18, 19], which is the product of the paternally imprinted CDKN1C gene, the expression of which is associated with the presence of maternal DNA [9]. According to data from the literature, as well as received results, by immunohistochemical investigation of the product of conception taken in early terms of short-term dysregulated pregnancies, by evaluating the immunoexpression of p57 in the villous cytotrophoblast, it was possible to distinguish two evolutionary subtypes of HM: complete and partial. At the same time, when evaluating the statistical tests, significant differences were established in the CHM vs PHM, CHM vs AS/AM groups, but not in the PHM vs AS/AM groups, results that do not contradict the literature data [20, 21].

Complete, partial, and non-molar molar lesions in the germinal compartment are established during histomorphological examination and frequently treated as inconclusive due to interobserver and intraobserver variability. This inconclusiveness in the differential diagnosis of the molar and non-molar profile is the result of the lack of certain morphological peculiarities in the choriovilar compartment [4, 5] and frequently presents diagnostic difficulties in the early period of pregnancy [6]. The evaluation of the trophoblastic epithelial proliferative profile has a priority importance in the diagnostic management, thanks to the variable deciphering of the histopathological lesions in early-term pregnancies, and thanks to the impact on the clinical management. Thus, the elucidation of an applicative marker for differential diagnosis, together with the evaluation of the proliferative pattern, remains a subject for studies.

Ki67 is a non-histone nuclear protein present in all active phases of the cell cycle, being virtually absent in quiescent (G_0) cells. Ki-67 protein expression is strictly associated with cell proliferation, therefore it can be used to determine dividing cells [10]. According to the bibliographic data, being a marker of proliferative activity, the expression of the Ki67 oncogene can also be applied in trophoblastic disease, including HM subtypes, with predictive value for the progression to a gestational trophoblastic neoplasia [22].

In the presented study, Ki67 immunoexpression was limited to villous cytotrophoblast, syncytium being negative. Ki67 expression in stromal cells was in most cases limited to a score of +1 in the group of abortions on social indications/ desire and a moderate score of +2 for molar pathology. The given results are in accordance with a series of studies, which denote the accentuated and differentiated immunoexpression in the villous cytotrophoblastic compartment [24]. The obtained data do not contradict the literature data. They denote the presence of different expression of Ki67 in molar and non-molar pathology, as well as in the CHM and PHM subtypes [23-25].

The histopathological features described in the literature as morphological criteria for differentiating molar and non-molar pathology remain uncertain due to interobserver and intraobserver variability. This discrepancy may also be determined by various technical factors, protocols and examination techniques, polymorphic tissue material. Overall, the immunohistochemical investigation by applying the anti-Ki67 antibody comes in handy as a complementary method in the differential diagnosis of molar pathology in early-term pregnancies.

Conclusions

Villous cytotrophoblast proliferative activity is high in the complete hydatidiform mole, and the immunoreactivity distribution index is highly positive and statistically true in the molar versus non-molar group. Immunohistochemical evaluation of Ki-67 protein in molar pathology is useful in differential diagnosis as a complementary method.

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EC designed the study, performed the laboratory work, interpreted the data; VP conducted the laboratory work, collected the material, interpreted the data; LS collected the material, interpreted the data; VF revised the manuscript; VD designed the study, conducted the laboratory work, interpreted the data, drafted the first version of the manuscript. All the authors reviewed and approved the final version of the manuscript.

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Conflict of interests. Nothing to declare.