ORIGINAL RESEARCHES

https://doi.org/10.52418/moldovan-med-j.65-2.22.01
UDC: 618.32+611.013

Evaluation of p57 expression in early disordered pregnancies with molar status vs non-molar ones

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Manuscript received September 21, 2022; revised manuscript November 30, 2022; published online December 20, 2022

Abstract

Background: The molar and non-molar lesions are determined during histomorphological examination and treated as inconclusive. The establishing of a marker by immunohistochemical investigations could influence the accuracy of the morphopathological diagnosis. The aim is evaluation of p57 immunoexpression in the trophoblastic germ compartment in molar and non-molar pregnancies.
Material and methods: Abortion products from 15 patients with hydatidiform mole, 18 pregnancies solved on social indications and 16 short-term disordered pregnancies were evaluated depending on the immunoexpresion of p57.
Results: The hydatidiform mole was classified based on anti-p57 immunoexpression into: complete hydatidiform mole – 8 cases (negative immunoexpression or expression in <10% of villous cytotrophoblast) and partial hydatidiform mole – 7 cases (positive expression in >10% of the villous cytotrophoblast). Basal deciduous and extravillous cytotrophoblasts were positive in 100% of cases and served as internal control. Hepatocytes were used as negative control. In the control group, the positive immunoexpression was attested in >10% of cases in the villous trophoblast.
Conclusions: Differential immunoexpression of p57 protein in the germinal cytotrophoblast allows subclassification of molar pathology into complete and partial forms, while not allowing the differentiation between partial hydatidiform mole and non-molar lesions. Immunohistochemical evaluation of p57kip2 protein in molar and non-molar pathology is useful in differential diagnosis as a complementary method.
Key words: anti-p57, fetal concept, hydatidiform mole, trophoblastic disease.

Cite this article
David V, Petrovici V, Saptefrati L, Fulga V, Carpenco E, Frant E. Evaluation of p57 expression in early disordered pregnancies with molar status vs non-molar ones. *Mold Med J*. 2022;65(2):5-12. https://doi.org/10.52418/moldovan-med-j.65-2.22.01.

Introduction

The morphological profile that is evaluated in short-term dysregulated pregnancies includes a heterogeneous group of choriovillar abnormalities accompanied by polymorphic proliferative and hydropico-cystic lesions in the germinal compartment of the fetal conceptus. The complete, partial and non-molar molar lesions are established most often during the histomorphological examination in early-term deranged pregnancies in the germinal compartment, being often treated as inconclusive. This inconclusiveness of the histomorphopathological diagnosis is the result of the masking of the particularities attested at the level of the choriovillary stroma, in common with the degree of proliferation of the villous trophoblast in early terms of pregnancy [1, 2]. The histomorphological peculiarities are often not obvious and do not help in establishing of the differential diagnosis in molar pathology, particularly in the early period of evolution [3], including, in the association of morphological lesions with a hydropic or cystic character [4]. Gersell D. et al. (2011) indicate that the differential diagnosis becomes even more unpredictable in the case of the addition of hydropic degenerative changes in the chorionic villi (15%-40%), being more frequently attested in more advanced pregnancies [4]. The molar phenotypic profile of the fetal conceptus is usually diploid and has androgenic origin, with the establishment of the complete molar monospermatic or dyspermatic form, through the loss of maternal chromosomes before or after fertilization. In the case of the partial hydatidiform mole, it represents a fertilization of a normal egg by two spermatozoa or by a diploid spermatozoon in most cases [5-7]. Also, despite the fact that most molar pregnancies are diploid or triploid, they are quite frequently associated with molar pregnancies with numerical and structural

anomalies, or with a twin pregnancy, where the second fetus develops normally [8].

Thus, the establishment of a marker by immunohistochemical investigations could influence the accuracy of the morphopathological diagnosis in molar pregnancies. The protein p57kip2 (p57) is expressed in pregnancies with non-molar, pseudomolar choriovillary phenotype and is not expressed in complete molar pregnancies [9, 10]. A series of studies using p57 protein demonstrate its effectiveness in identifying the complete molar profile (by negative immunoexpression in the germinal compartment) vs partial hydatidiform mole, evaluated with positive p57 expression in most cases [11]. Therefore, the aim of the study was the differential evaluation of p57 immunoexpression in the trophoblastic germinal compartment in molar versus non-molar pregnancies.

Material and methods

The tissue samples were taken from the material obtained after medical abortion from 15 patients with short-term pregnancies (3-12 weeks) in the Mother and Child Institute, Perinatal Center of level III, during 2019-2021. All the samples were diagnosed as hydatidiform mole (group I). The age of the patients in this group varied between 17-47 years (28.4±9.36). All patients were previously examined by ultrasound and in 5 cases the molar ultrasound character was established.

The control group (group II) included the material taken from the germinal sac of pregnancies solved on social indications (SA) from 8 patients, aged between 22-40 years (30.5±5.6) and pregnancies solved on medical indications (MA) (16 patients) in the short term (with the diagnosis of spontaneous abortion or stagnant pregnancy, in the presence of hydropic and/or dysplastic morphopathological lesions), age between 23-41 years (31.3±6.7). Clinical data were obtained from each patient's medical records. The current research is part of a larger study of disordered early-term pregnancies.

Primary processing. The tissue material was collected fastly at the Obstetrics Department after the medical intervention, with rapid fixation in 10% formalin (pH 7.2-7.4) to reduce the risk of early lysis of tissue material and growth of bacterial flora. The time of fixation in formalin was no 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 placed on positively charged slides (APTACA, Italy).

Histological method. Sections were stained by the classical conventional hematoxylin-eosin (H.E) method, using Mayer's hematoxylin (HEMM-36/21, BIOGNOST, Slovenia) and eosin Y 1% (EOY10-35/21, BIOGNOST, Slovenia). Sections for HE 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. Immunohistochemical assays were performed using the manual procedures adopted for the anti-p57 antibody (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 the Novolink™MaxPolimer detection system, Leica, (RE7280-K) [12]. Details regarding the antibody can be found in Table 1. Deparaffinization was performed in two toluene baths (code UN1294, Sigma-Aldrich), 5 minutes each, followed by a mixed bath of toluene and alcohol 99.9% (code 06-10077F) for 5 minutes, then – 2 baths of absolute alcohol 99.9% with rehydration in distilled water. In order to unmask the epitopes, the sections were exposed to Na citrate solution, pH 6.0, in a water bath at 95°C-96°C, with a total pretreatment and posttreatment time of 60 minutes. Incubation of the sections with the primary antibody was followed by blocking of endogenous peroxidase by applying the Peroxidase-Blocking solution for 5 minutes, and DAB (3,3'-diaminobenzidine) was applied as a chromogenic substrate for 5 minutes. Nuclei were counterstained with Leica hematoxylin (RE7164). The final product of the reaction was colored brown with a nuclear pattern. Then, the panel of histological slides was subjected to the procedure of dehydration and clarification in absolute alcohol, one shot of mixed alcohol and toluene, and three shots of toluene, each exposure being 5 minutes. The final procedure was mounting the slides with BMC-100 solution. In the manual immunohistochemical staining procedure, the Sequenza™ Immunostaining Center was applied using the Thermo Shandon Coverplate.

Table 1. Antibody 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™-MaxPolimer, Leica / 20 min

Microscopic evaluation. The positive expression of p57 in the choriovillar and gestational deciduo-endometrial germinal compartment was determined based on nuclear staining in decidual cells 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 the cells, in the concomitant presence of the positive internal control. Chorionic villi and decidual plates from normal human placenta served as positive ex-

ternal control. The negative external control was represented by the liver (negative immunoreaction), being included in each research set. Cytoplasmic expression was considered non-specific. The quantification method was applied after Gupta et al. [13] and was performed with the Axio Imager A2 microscope (Carl Zeiss, Germany) equipped with an AXIOCam MRc5 recording camera.

Statistical analysis. The results of the study were stored in the Access 2007 database (Microsoft Office 2007). Statistical processing was performed using Winstat 2012.1 software (R. Titch Software, Bad Krozingen, Germany) The threshold value for statistically significant results was $p \leq 0.05$.

Results

The study was carried out on a batch of 15 cases of short-term disordered pregnancies with the morphopathological diagnosis of hydatidiform mole, the age of the patients varying between 17-47 years (28.4±9.3). Most of the patients were between 17 and 35 years old (11 cases/73.3%). In 3 cases/20% the age was greater than 35 years. The cases were classified based on the gestational period as follows: the group with the gestational period of 8 weeks (6/40.0% of cases), 9 weeks (4/26.7% of cases), 10 weeks (2/13.3% of cases), 7 and 12 weeks – 1 case each (6.66%). Data regarding age and gestational period were missing in 2 cases. During the primary investigation of the fetal conceptus from the group with disordered early-term pregnancies (basic group), it corresponded to type V fetal conceptus (molar-hidatiform fetal conceptus, MHFC) in 8 cases (53.3%) and was characterized by macroscopic multicystic lesions in the choriovilar germinal compartment, with variable sizes from 0.15 to 0.6 cm, with fluid-transparent content (fig. 1a). In the rest of the cases, the abortive product corresponded to the disorganized fetal concept of type VI (growth disorganized embryos, GDE), consisting of various categories of tissue plates (thickened and thinned), germinal sac (fragmented, mush-like) (fig. 1b). The volume of the abortive product was abundant in 100% of cases, with dimensions of 5.0x6.0x2.0cm, including the hemorrhagic component organized in blood clots. Embryo fragments were attested only in one case (6.66%).

In the control group, most patients were between 17 and 35 years (25 cases/80.6%) or older than 35 years (6/19.35%). The cases were classified based on the gestational period as follows: the group with a gestational period of 6-9 weeks –

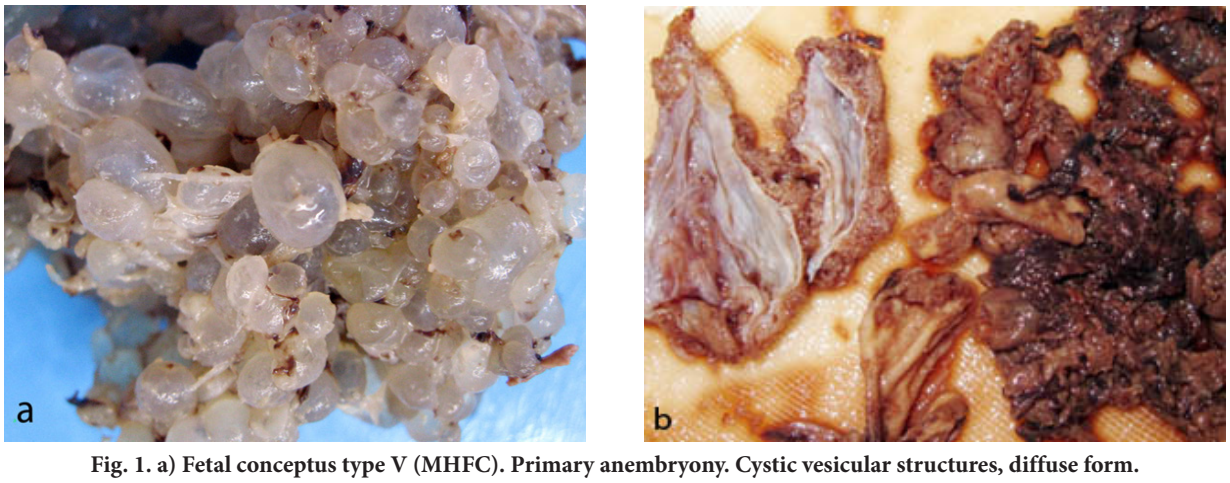

Fig. 1. a) Fetal conceptus type V (MHFC). Primary anembryony. Cystic vesicular structures, diffuse form. b) Fetal conceptus type VI (GDE). Secondary anembryony. Decidual and choriovillous tissue plates. Damaged germinal sac

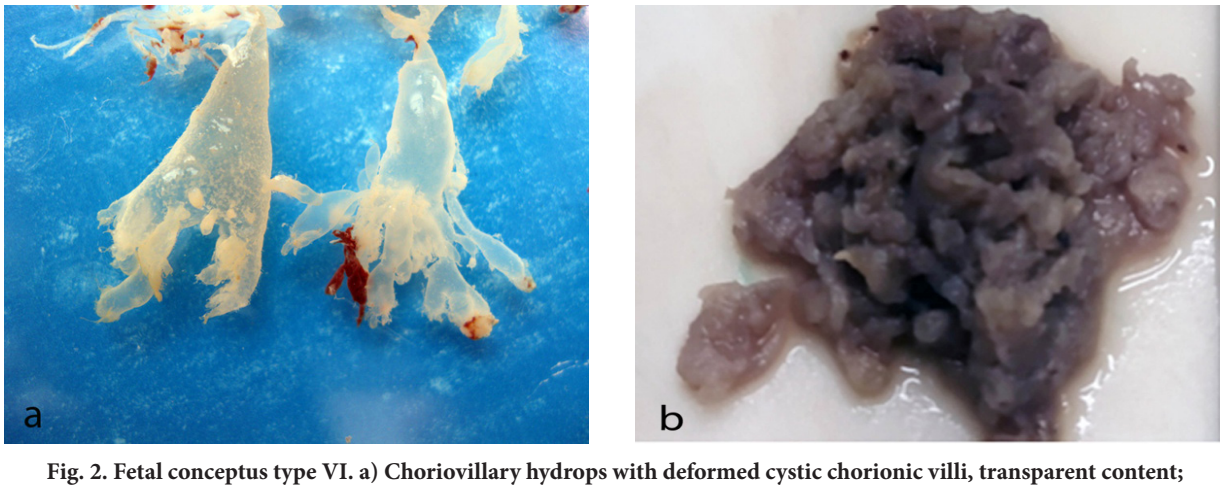

Fig. 2. Fetal conceptus type VI. a) Choriovillary hydrops with deformed cystic chorionic villi, transparent content; disordered dichotomous division; b) Mush-like appearance of the choriovillar product

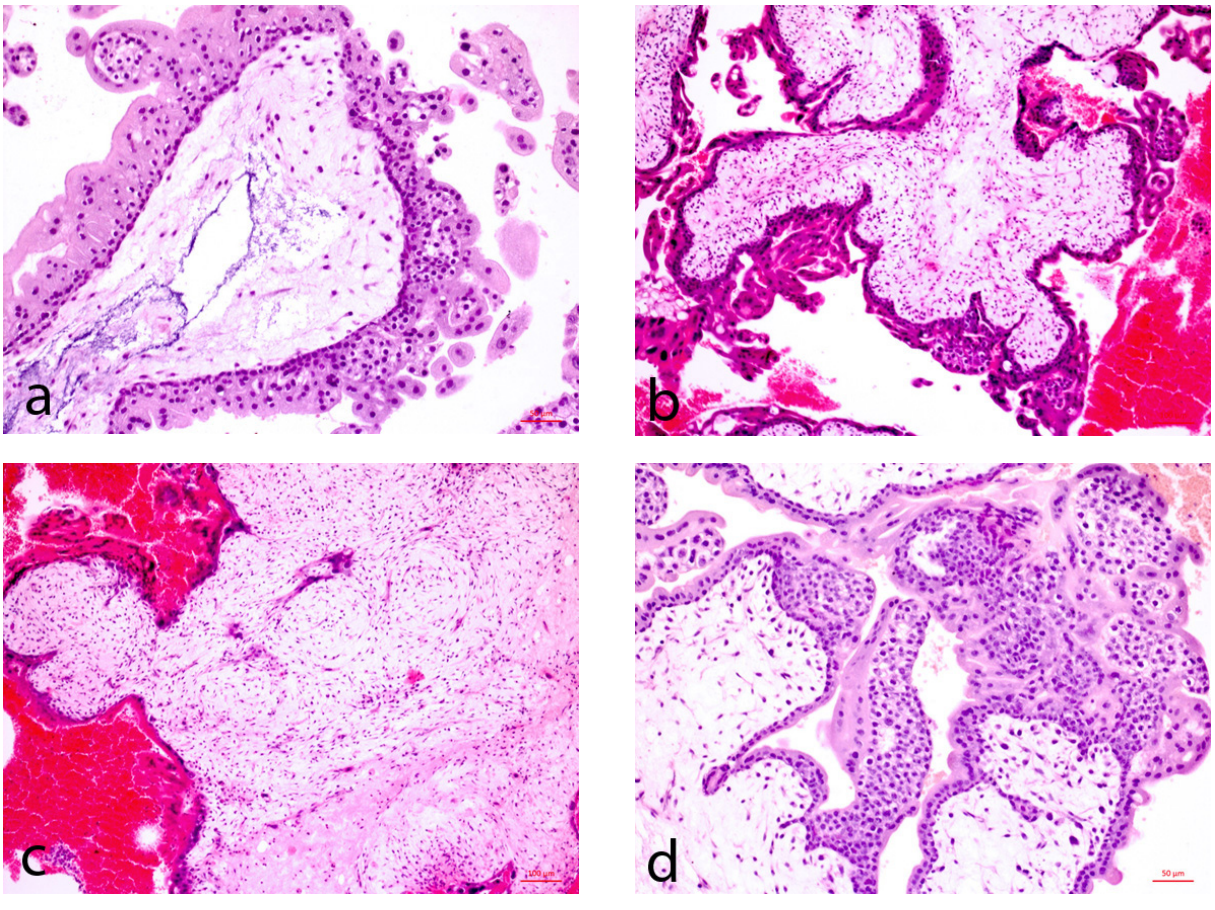

Fig. 3. Fetal conceptus type V (MHFC), complete type: a, d) Trophoblastic proliferation: mixed and cytotrophoblastic form; a, b) Stromal cystic structures with edema, hypo/hypercellularity; c) Stromal dysplasia with trophoblastic inclusions; a-d) Stromal anavascularization. HE, × 100, 400

22 cases (70.96%), and the group with a gestational period of 10-12 weeks – 9 cases (29.04%). During the primary investigation of the fetal conceptus obtained from 31 patients, in 21 cases (67.74%) the fetal conceptus was type VI (GDE), including embryo fragments in 5 cases (16.13%), and in 10 cases (32.25%) it had a mush-like appearance. The macroscopic peculiarities were mainly common, including a fragmented germinal sac, mush-like appearance, thinned and thickened tissue plates with various characters of expression, with or without embryo fragments. In some cases, lesions with a cystic appearance were associated, having variable sizes (from 0.4-1.9 cm) containing serous and transparent liquid (fig. 2). The volume of the abortive product was variable, predominantly abundant, including a hemorrhagic component in the form of blood clots or in a dispersed form with a mottled appearance.

When comparing the clinical diagnosis with the morphological one, in 33.3% of cases, the primary clinical diagnosis contained the notion of hydatidiform mole. In the rest of the cases, the clinical data were evaluated as endometrial gland hyperplasia or stagnant pregnancy.

By applying the complementary histological investigation by hematoxylin-eosin with the evaluation of histopathological lesions, the complete hydatidiform mole was observed in 8 cases (53.35%) out of 15 (fig. 3), and the partial one – in 7 cases (46.6%) (fig. 4).

In the group of early disturbed pregnancies (n=16), morphological lesions corresponding to the hydropic pattern at the level of the villous chorion were established in 10 cases (62.5%), and the dysplastic component – in 6 cases (37.5%) (fig. 5, 6).

Subsequently, all cases were subjected to immunostaining with anti-p57 (nuclear pattern), with the evaluation of two distinct compartments: germinal site (choriovilar) and gestational (deciduo-endometrial). In the molar group, the villous cytotrophoblastic site was positive in 7 cases (46.7%) and negative or <10% positive in 8 cases (53.3%) (fig. 7).

In the control group with pregnancies solved on social indications and or disordered at early term with hydropic and dysplastic stromal component, anti-p57 nuclear immunoexpression in the villous trophoblastic compartment was attested in 100% of cases (fig. 8).

In all cases, the internal positive control was attested in the region of the extravillous trophoblast, associated with the intense expression in the cells of the basal decidua (fig. 9 a, b). Hepatocytes served as external negative control (fig. 9 c).

Data analysis revealed statistically significant correlations of p57 expression in the following groups: CHM vs PHM ($p<0.003$), CHM and SA/MA ($p<0.001$). When analyzing the results of the given study, a series of correlations were found in relation to the age of the patient. This criterion correlated positively and statistically significantly with the term of gestation in the group of pregnancies with molar pathology: CHM ($rs=0.89$, $p<0.02$) and PHM

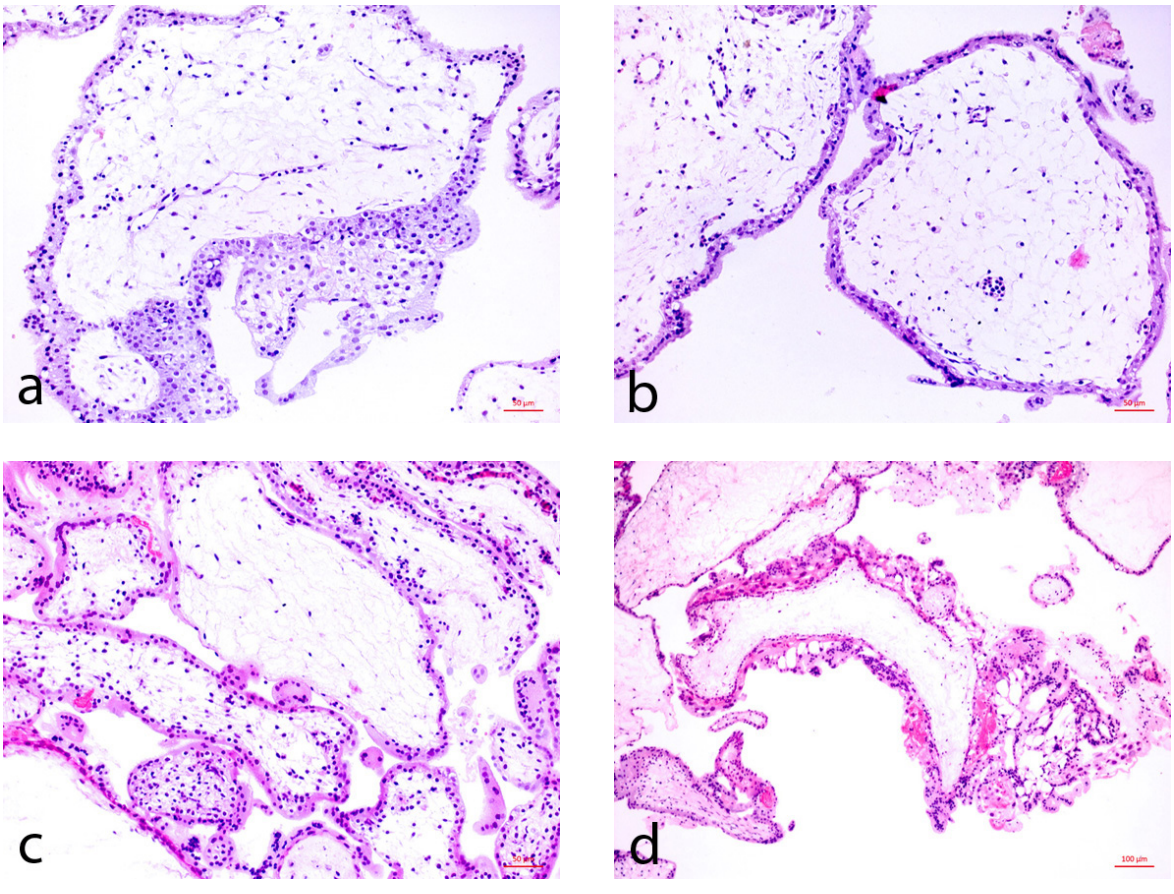

Fig. 4. Fetal conceptus type V (MHFC), partial type: a) Focal trophoblastic proliferation with anemic vascular component; b) Trophoblastic atrophy with absence of microvilli, vascular component with intravascular nucleated erythrocytes; c) Clusters of deformed villi with diverse stromal cellularity; d) chorionic villi with multifocal trophoblastic proliferation vs villi with acellular, hydropic stroma and trophoblastic atrophy. HE, × 100, 400

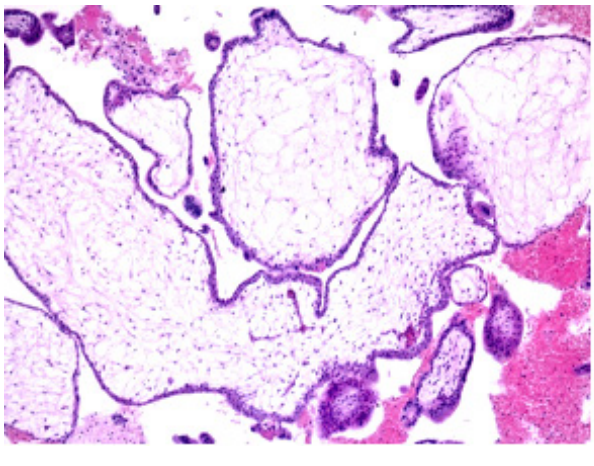

***Fig. 5.* Fetal conceptus type VI (GDE): Vilar hydrops. Hydropic/cystic choriovillar component. HE. × 100**

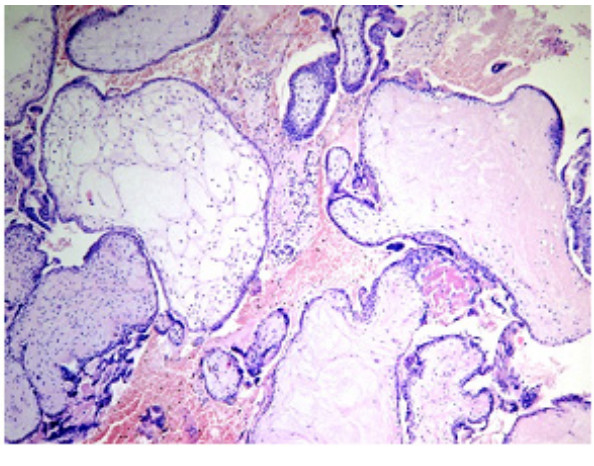

Fig. 6. Fetal conceptus type VI (GDE): Vascular stromal mesenchymal dysplasia. HE; × 100.

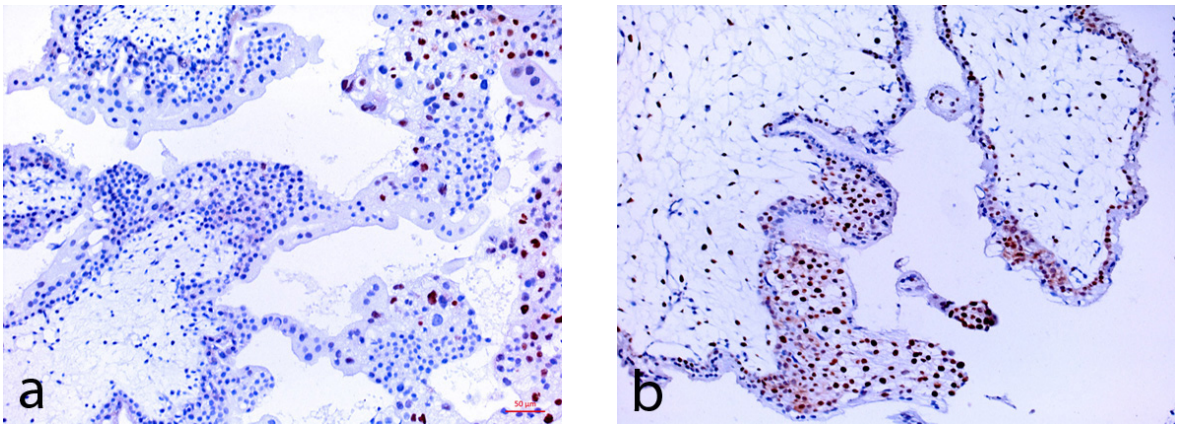

Fig. 7. Fetal conceptus type V (MHFC): a) CHM. Negative villous trophoblast immunoexpression. Intense nuclear immunoexpression in the extravillous trophoblast (internal control). Mixed cyto-syncytiotrophoblastic proliferation; b) PHM. Intense positive villous cytotrophoblast immunoexpression. Cytotrophoblastic proliferation. Immunoreaction for anti-p57, DAB

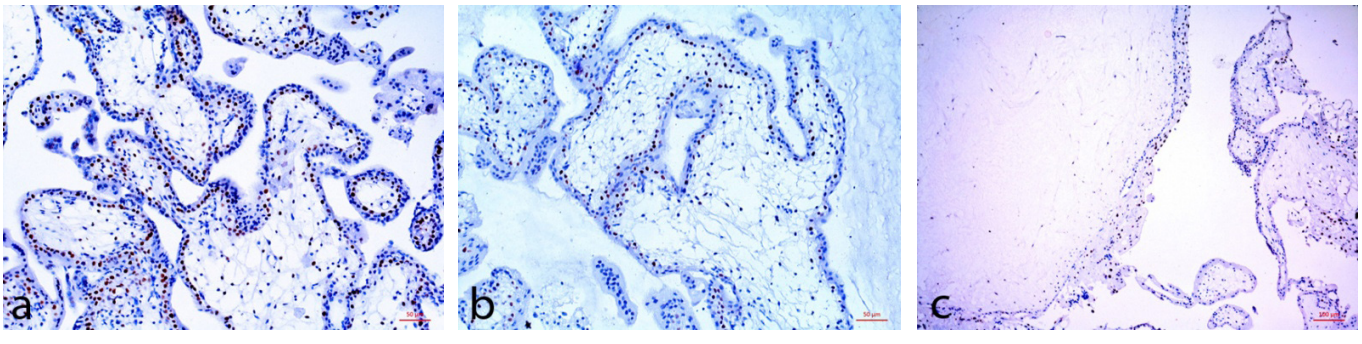

Fig. 8. Fetal conceptus type VI (GDE): a) Social abortion; b) Vilar hydrops; c) Mesenchymal dysplasia; Intense positive villous trophoblast immunoexpression. Immunoreaction for anti-p57, DAB

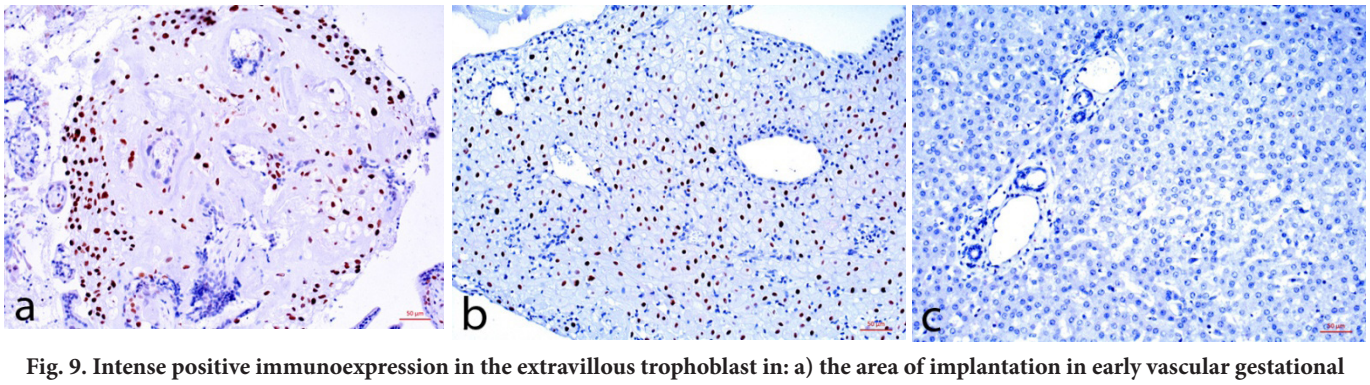

Fig. 9. Intense positive immunoexpression in the extravillous trophoblast in: a) the area of implantation in early vascular gestational conversion, b) the decidualocytes of a decidual plate (positive internal control); c) negative immunoexpression in hepatocytes (positive external control). Immunoreaction for anti-p57, DAB

(rs=0.75, p<0.04). The value of this marker, however, did not correlate statistically significantly in the group of control pregnancies and those that evolved with spontaneous abortion or stagnated in evolution (rs=0.17, p>0.28).

Discussion

Molar trophoblastic pathology represents a nosological entity describing an early placentation with choriovillary abnormalities of a proliferative and hydropic-cystic-malformative nature in the germinal compartment of the fetal conceptus in the case of abnormal fertilization. The histomorphological peculiarities are often confusing in establishing of the differential diagnosis of the molar pathology (CHM vs PHM) in the early period [3], because they also describe morphological lesions with a hydropic or cystic character [4].

Cytogenetic and ploidy analysis described the molar hydatidiform profile by several features. CHM is usually diploid and presents a 46XX karyotype and less often 46XY. Ocasionally, it can be tetraploid, including a small

percentage of cases with the biparental genome [7]. Androgenic origin is characterized by the lack of the maternal genome, as a result of the reduplication of a haploid paternal genome or being obtained by dispermatic fertilization. PHM presents as a triploid genome, in most cases with a 69XXX or 69XYY karyotype. The tetraploid genome includes a maternal and paternal haploid genome, paternal one being reduplicated as a result of dispermatic fertilization [6].

Thus, the evaluation of a marker through immunohistochemical investigations can influence the adjustment of the particularities of the morphopathological profile in molar pregnancies. In this context, the protein p57kip2 (p57) is the protein product of the paternally imprinted CDKN1C gene, the expression of which is associated with the presence of maternal DNA. It is present in pregnancies with a non-molar, pseudomolar choriovillous phenotype and absent in complete molar pregnancies [9, 10].

Kinara M. et al. (2005), Sasaki S. et al. (2015) highlight the impact of differential p57kip2 immunoreactivity in molar pathology of androgenic origin [14, 15].

In the present study it was possible to differentiate 2 forms of hydatidiform mole (CHM vs PHM) by evaluating the immunoexpression of p57 in the villous cytotrophoblast. It should be mentioned that p57 did not express at the site of the villous cytotrophoblast in all cases, which allowed to evaluate these pathological forms in the context of differential immunostaining. The results obtained in the given study are consistent with a series of studies according to which the complete hydatidiform mole does not contain a maternal genomic component, the application of the immunohistochemical reaction with p57 being important in the differential diagnosis of the molar pathology by indirectly identifying the presence of the maternal genome [16-18]. The authors mention that the application of the p57 protein demonstrates its effectiveness in identifying the complete molar profile by negative immunoexpression in the germinal compartment vis a vis the partial hydatidiform mole, which is p57 positive in most cases [11]. This divergent expression of p57 (positive or negative) in the germinal site of complete or partial hydatidiform mole denotes the loss or preservation of the maternal copy of the DNA [15]. In the systematic review and meta-analysis, Madi J. M. et al (2018) found a summary sensitivity of 0.984 (95% CI: 0.916-1.000) and a specificity of 0.625 (CI 95%: 0.503-0.736), with a curve diagnostic performance below 0.980 [19]. Ronnett B. M. (2018) indicates that the immunohistochemical evaluation of p57kip2 is practical and can be applied in histopathological practice as an adjunctive investigation in addition to the histomorphological one to help in the differential diagnosis of MHC [20].

When evaluating the statistical tests, significant differences were established in the groups between CHM vs PHM, CHM vs SA/MA, but not in PHM vs SA/MA, results that do not contradict with the literature data [21-25]. The given study highlighted that the majority of patients with the diagnosis of molar pathology were between 17 and 35 years old and the gestational period was within the limits of 6 to 9 weeks. Similar studies in the given field denote a minimal variability of the age interval [19].

In clinical practice, the differential evaluation of the hydatidiform molar profile is important. The most important reason for differentiating the subtypes of molar pathology in early terms is the possibility of the association of trophoblastic disease or choriocarcinoma [22]. Hydropic abortion is completely benign, while hydatidiform mole has a significant risk of transformation into persistent gestational trophoblastic disease, the incidence of which is higher in patients with complete hydatidiform mole (10-30%) vs patients presenting a partial molar form (0.5-5 %) [25].

Conclusions

Differential immunoexpression of p57 protein in the germinal cytotrophoblast allows subclassification of molar pathology into complete and partial form. It does not allow the differentiation between partial hydatidiform mole and non-molar lesions. Immunohistochemical evaluation of p57kip2 protein in molar and. non-molar pathology is useful in differential diagnosis as a complementary method.

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

Funding
This work was carried out as a part of the state project "Morphological approach by conventional, histo- and immunohistochemical methods of the peculiarities of the pathological profile of early placentogenesis in short-term disordered pregnancies". Project code 20.80009.8007.17.

Ethical approval and consent to participate
No approval was required for this study.

Conflict of interests
There are no conflicts of interest.