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***“Expression of pro-thrombotic markers in placentas
from complicated and uneventful pregnancies”***

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“Expression of pro-thrombotic markers in placentas from complicated and uneventful pregnancies”

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INTRODUCTION

The hypertensive disorders of pregnancy remain a leading cause of maternal and perinatal morbidity and mortality in Europe and North America. These disease challenge the medical and obstetric skills of the health care team. Decision as to the possible use and appropriate choice of pharmacologic agents require not only an understanding of the pathophysiology of the hypertensive disorders and a recognition of the pharmacokinetic changes occurring during pregnancy but also an appreciation of the possible fetal effects of such therapeutic agents.

Obstetric management demands meticulous maternal observation and use of tests of fetal-placental function and fetal maturity in order to weigh maternal risks and the risks to the infant of intrauterine versus extrauterine existence. The management of elevated blood pressure and the impact of the disorder on the mother and fetus depend on whether hypertension antedated the pregnancy or appeared as the marker of pregnancy-specific vasospastic syndrome. The hypertensive disorders of pregnancy were for many years called toxemias of pregnancy, a term that originally included even hyperemesis gravidarum and acute yellow atrophy of the liver. One of the difficulties in interpreting studies of the hypertensive disorders of pregnancy is the inconsistency of terminology. Several systems of nomenclature are used around the world. The system prepared by the National Institutes of Health (NIH) working group on hypertension in pregnancy (1) although as imperfect as all such systems, has the advantage of clarity and is available in published form to investigators throughout the world.

This classification is as follows:

- 1) Chronic hypertension
- 2) Preeclampsia-eclampsia
- 3) Preeclampsia superimposed upon chronic hypertension
- 4) Gestational hypertension

For mild-to-moderate pregnancy hypertension, maternal risks are small, and there may be adverse perinatal consequences of blood pressure normalization.

Chronic hypertension

Chronic hypertension is defined as hypertension that is present and observable prior to pregnancy or that is diagnosed before the 20th week of gestation. Hypertension is defined as a blood pressure greater than 140/90 mmHg. Hypertension for which a diagnosis is confirmed for the first time during pregnancy and which persist beyond the 84th day postpartum is also classified as chronic hypertension.

Preeclampsia and eclampsia

The diagnosis of preeclampsia is determined by increased blood pressure accompanied by proteinuria. Pre-eclampsia complicates 3 to 8% of pregnancies and usually this disease is defined on the basis of new onset hypertension and albuminuria developing after 20 weeks of pregnancy. Diagnostic blood pressure increases are either a systolic blood pressure of greater than or equal to 140 mmHg or a diastolic blood pressure of greater than or equal to 90 mmHg. It is recommended that gestational blood pressure elevation be defined on the basis of at least two determinations. The repeat blood pressure should be performed in a manner that will reduce the likelihood of artefact and/or patient anxiety. (2) Absent from the diagnostic criteria is the former inclusion of an increment of 30 mmHg systolic or 15 mmHg diastolic blood pressure, even when absolute values are below 140/90 mmHg. This definition was excluded because the only available evidence shows that women in this group are not likely to suffer increased adverse outcomes (3) Notheless, women who have a rise of 30 mm Hg systolic or 15 mmHg diastolic blood pressure warrant close observation, especially if proteinuria and hyperuricemia are also present. Proteinuria is defined as the urinary escretion of 0,3 g protein or greater in a 24 hour specimen. This will usually correlate with 30 mg/dL or greater in a random urine determination. Because of the discrepancy between random protein determinations and 24-hour urine protein in preclampsia, it is recommended that the diagnosis be based on 24 hour urine specimen if at all possible, or if this is not feasible, it should be based on a timed collection corrected for creatinine excretion (2). Preeclampsia occurs as a spectrum but is arbitrarily divided into mild and severe forms. The terminology is useful for descriptive purposes.

The diagnosis of severe preeclampsia is confirmed when the following criteria are present:

- ✓ Systolic blood pressure of 160 mmHg or greater, or diastolic pressure of 110 mmHg or greater
- ✓ Proteinuria of 2 g or more in 24 hours (2 or 3 plus on qualitative examination)
- ✓ Increased serum creatinine (greater than 1,2 mg/dL unless known to be previously elevated)
- ✓ Persistent headache or cerebral or visual disturbances
- ✓ Persistent epigastric pain
- ✓ Platelet count less than 100,000/mm³ and/or evidence of microangiopathic haemolytic anemia (with increased lactic acid dehydrogenase)

Eclampsia is the occurrence of seizures in a preeclamptic patient that cannot be attributed to other causes. Edema occurs in too many normal pregnant women to be discriminant and has abandoned as a marker in preeclampsia by the National High Blood Pressure Education Program and by other classification schemes.

Preeclampsia superimposed on Chronic Hypertension

Preeclampsia can occur in women who are already hypertensive and the prognosis for mother and fetus is much worse. Distinguishing superimposed preeclampsia from worsening chronic hypertension tests the skills of the clinicians. The suspicion of superimposed preeclampsia mandates close observation with delivery indicated by the overall assessment of maternal-fetal health. The diagnosis of superimposed preeclampsia is highly likely:

1. In women with hypertension and no proteinuria early in pregnancy (prior to 20 week's gestation) and new-onset proteinuria, defined as the urinary excretion of 0,3 g protein or more in a 24h specimen
2. In women with hypertension and proteinuria prior to 20 weeks' gestation:
 - A sudden increase in proteinuria – urinary excretion of 0,3g protein or more in a 24-hour specimen or two dipstick-test result of 2+ (100mg/dl) with the values recorded at least 4 hour apart with no evidence of urinary tract infection
 - A sudden increase in blood pressure in a woman whose blood pressure has previously been well controlled
 - Thrombocytopenia (platelet count lower than $100000/\text{mm}^3$)
 - An increase in ALT or AST to abnormal levels

Gestational Hypertension

The woman who has blood pressure elevation detected for the first time during pregnancy, without proteinuria is classified as having gestational hypertension. This non-specific term includes women with preclampsia syndrome who have not yet manifested proteinuria as well as women who do not have the syndrome. The final differentiation that the woman does not have preclampsia syndrome is made only post-partum. If preclampsia has not developed and blood pressure has returned to normal by 12 weeks post-partum the diagnosis of transient hypertension is made. If blood pressure elevation persists, the woman is diagnosed as having chronic hypertension.

There are some problem with the classification; the degree of blood pressure elevation that constitutes gestational hypertension is controversial. Because average blood pressure in women 10-20 years is 120/60 mmHg, the standard definition of hypertension (blood pressure greater than 140/90 mmHg) is judged by some investigators to be too high. NHBPEP report that women with increased BP greater than 30 mmHg systolic or 25 mmHg diastolic must be observed closely even if absolute blood pressure has not exceeded 140/90 mmHg.

Preeclampsia has a clinical spectrum ranging from mild to severe forms and then potentially to eclampsia. Affected patients do not catch eclampsia or the severe forms of preclampsia but rather progress through this spectrum. In most cases, progression is slow and the disorder may never proceeded beyond mild preclampsia. In other patients, the disease can progress more rapidly chancing from mild to severe over days to weeks. In the most serious cases, progression can be fulminant with mild preclampsia evolving to severe preclampsia or eclampsia over hours to days.

HELLP syndrome

In Hellp syndrome the acronym stands for Hemolysis, Elevated liver enzymes and Low platelets. This series of findings defines a reasonably consistent syndrome. Women with the Hellp tend to be older and are more likely Caucasian and multiparous. In many cases they are not hypertensive. (4)

Preclampsia: risk factors

Preeclampsia is a common pregnancy disorder that originates in the placenta and causes variable maternal and fetal problems. The disorder is unique to pregnancy, characterized by poor perfusion of many vital organs and completely reversible with the termination of pregnancy. Problems still arise owing to the approaches in the management of PE; the successful management of PE requires an understanding of the pathophysiologic changes in this condition and the recognition that signs of preeclampsia are only signs, not causal abnormalities. Preeclampsia occurs in about 4% of pregnancies. It would be useful to be able to identify the women at greater risk. As with all diseases there are no “typical” patients. Epidemiologic findings do indicate, that certain characteristics are more common in women who develop preeclampsia. The most important is nulliparity. At least two thirds of cases occur in women during the first pregnancy. In some studies the PE was more common among women of lower socio-economic status, but other studies didn't find established correlation. Eclampsia is clearly a disease of women of lower socioeconomic status; it is preventable by careful obstetric observation and prompt delivery. The lack of availability and use of good-quality obstetric care to indigent women is undoubtedly a major factor in the increased incidence of eclampsia. There is a relationship between the extremes of childbearing age and the onset of eclampsia. Because most pregnancies particularly the first occur in young women, most cases occur in this group. Some studies don't find a correlation with the younger women and point the vision on older women. The incidence can vary in the different countries of the world; the relationship with the race is equally difficult to evaluate. In the Collaborative Study the incidence of PE was higher in blacks. (5) This risk factor was observed especially in

nulliparous women (6) Analysis from a National Institutes of Health study of aspirin prophylaxis for low-risk pregnancies also failed to identify race as a risk factor (7). A characteristic of preclampsia-eclampsia is the tendency of the condition to occur in daughters and sister of women with a history of PE. The inheritance pattern was not best explained by simple mendelian inheritance. There is also a contribution of the fetal genome to preclampsia. Men who have fathered preeclamptic pregnancies are more likely to father preeclamptic pregnancies with new partners than the men who have never been fathers in preeclamptic pregnancies (8) In addition men born in preeclamptic pregnancies are more likely to be fathers in preeclamptic pregnancies than the who are born in nonpreeclamptic pregnancies. (9) Immunologic differences, features that compromise implantation and an increased sensitivity to respond to the systemic insult caused by reduced placental perfusion are factors presupposed for etiologic origin of PE. Some Human leukocyte antigen types are more common in the mothers and the fetus from preeclamptic pregnancies than others. Gene variants potentially leading to aberrations of endothelial function are more common in preeclamptic women. Mutations leading to increased risk factors for later-life cardiovascular disease, including function perturbing mutations of lipoprotein lipase genes and methylene tetrahydrofolate reductase are associated with PE. (10-11) With all of these genetic polymorphism the result are inconsistent, being associated in some but not other populations supporting the heterogeneity of PE. Some medical disorder as diabetes also predispose to PE (50%). Preeclampsia is also more common in women with hypertension antedating pregnancy (20%). (12) Obesity is also risk factor. In the NIH study of aspirin for low-risk pregnancies, a dose relationship of obesity and PE was present. (5) The incidence of PE

increased with the magnitude of obesity. The mechanism of the increased risk could be related to increased insuline resistance, as PE is also more common in gestational diabetes (13). The relationship among obesity, insuline resistance and PE are part of an interesting relationship of PE to the metabolic or insulin resistance syndrome. This syndrome predispose to cardiovascular disease in later life and consist of obesity, hypertension, dyslipidemia (increased triglycerides and LDL cholesterol, decreased HDL cholesterol) and increased uric acid. All of these changes are present in women with PE. (14) Twin pregnancies also increase the risk of PE. In case of PE occurring before 24 th week' gestation, hydatidi-form mole should be suspected.

IUGR

The most common definition of Intra uterine growth retardation is a fetus whose weight is below the tenth percentile for gestational age. This definition, however, is not universally accepted. Some authors may define it as a fetal weight below the fifth or third percentile. Abdominal circumference (AC) below the tenth or fifth or third percentile and lack of normal growth of the AC on serial examinations are other definitions. Others may categorize growth disturbance based on absolute weight at birth, such as less than 2500 or less than 1500g. However this obscures the distinction between smallness owing to prematurity and that owing to growth restriction. Causes and associations are Maternal, uterine placental, fetal, infection and teratogens. Once underlying fetal anomaly or aneuploidy is excluded, the most common associations are with maternal hypertension and a history of IUGR in previous pregnancy. Underlying uterine-placental dysfunction is a commonly evoked cause for otherwise unexplained fetal IUGR. Uterine placental dysfunction has been correlated with a range of pathologic findings, including smaller placentas, increase in the thickness of tertiary stem villi vessels wall and decrease in lumen circumference. Also confined placental mosaicism has been found to carry a higher risk of IUGR and adverse outcome including fetal death (15) Uterine placental dysfunction produces fetal hypoxia, which result in subnormal growth, oligohydramnios and alterations in blood flow. The strong association of growth restriction with still-birth support the need for early recognition of growth restriction. The primary means of detecting IUGR fetuses is demonstration of fetal weight to be less than the tenth percentile. The predictive value of true IUGR is further increased in association with oligohydramnios or abnormal Doppler studies. There are two forms of IUGR: the

symmetric and the asymmetric. Symmetric IUGR has been used to describe a growth pattern when all biometric measurements appear affected to same degree. The asymmetric IUGR has been used to characterize a smaller AC compared to other growth parameters. Asymmetric IUGR would then show abnormal ratios such as HC/AC ratio or FC/AC ratio. (16) The term symmetric IUGR was suggested as more likely to reflect underlying fetal condition including aneuploidy. The term asymmetric IUGR reflected underlying uterine placental dysfunction. The IUGR condition is at higher risk for perinatal morbidity and mortality and the risk rising with the severity of the growth restriction. The diagnosis of IUGR is in part dependent on an accurate evaluation of gestational age. The incidence varies according to the population under examination, the geographic location, the standard growth curves used as reference and the percentile chose to indicate abnormal growth. Approximately one fourth to one third of all infants weighing less than 2500g at birth have sustained IUGR. Approximately 4-8% of all infants born in developed countries and 6-30% in developing countries are classified as growth restricted. (17) IUGR is usually associated with a small placenta; the its mean surface and the capillary surface area are reduced, implying a diminished diffusing capacity. Cytotrophoblastic hyperplasia, thickening of the basement membrane, placental infarction and chorionic villi are commonly present in placentas from pregnancies complicated by maternal vascular disease and IUGR. (18) More recently it has been reported that trophoblastic apoptosis is increased in IUGR, the mechanism being unknown. (19) The terminal villi are maldeveloped in IUGR pregnancies when and-diastolic flow is demonstrated. Absent end-diastolic flow shows more occlusive lesions of the intraplacental vasculature than when end-diastolic flow is present.

Vascular change in the placenta Site during pregnancy

In normal pregnancy the spiral arteries increase greatly in diameter. Morphologically the endothelium is replaced by trophoblast and the internal elastic lamina and smooth muscle of the media are replaced by both trophoblast and an amorphous matrix containing fibrin. These changes occur originally in the decidual portion of the spiral arteries but extend into the myometrium as pregnancy advances and can even involve the distal portion of the uterine radial artery. The basal arteries are not affected. These morphologic changes are considered to be a vascular reaction to trophoblast, that results in increased perfusion of the placenta site. In placental site vessels of women with PE, the normal physiologic changes do not occur or are limited to the decidual portion of the vessels; myometrial segments of spiral arteries in myometrium retain the non pregnant component of intima and smooth muscle and the diameter of these arteries is about 40% that of vessels in normal pregnancy. In addition some spiral arterioles in decidua and myometrium and some basal and radial arterioles are affected by a change termed acute atherosclerosis. The affected vessels are necrotic and the usual components of the vessels wall are replaced by amorphous material and foam cells. This lesion is best seen in basal arteries because these arteries do not undergo the normal changes of pregnancy. It is also present in decidual and myometrial spiral arteries and can progress to vessel obliteration. The obliterated vessels correspond to areas of placental infarction. Although some believe that this change occurs only in PE, others hold that it is present in placental site vessels in pregnancies with IUGR and without clinical evidence of PE. This finding indicates that PE is a disorder of placentation and that characteristic pathologic changes have preceded the clinical presentation of this disorder. The etiology of the decidual vascular lesion is

not known. The appearance of these vessels is somewhat similar to that of vessels in transplanted kidneys that have undergone rejection. This is suggestion of an immunologic etiology.

The *placenta* is a unique organ with dual blood circulation functioning throughout fetal development. The architecture and functions of the placenta, where maternal blood flows in the intervillous space, present haemostatic problems, mainly the risk of haemorrhage. Placental trophoblasts express and produce coagulation components, participating not only in haemostasis, but also in the placental vascular development and differentiation. The expression of tissue factor (TF), membrane phosphatidylserine and fibrin render the trophoblasts pro-coagulant, thus compromising the risk of bleeding while exposing the placenta to pro-thrombotic risks. Local inhibitory mechanism TFPI-1 and TFPI-2, thrombomodulin, annexin V and the fibrinolytic system-limit coagulation activation and fibrin deposition. Pregnancy complications have been associated with abnormalities in the functions of these inhibitors. Haemostatic processes in placental cells change throughout gestation and are affected by the changing requirements of the organs.

Although most systemic coagulation proteins are produced by the liver, regulation of haemostasis is achieved by cellular membrane components or by locally expressed or secreted proteins. TF, the main activator of coagulation, is a glycoprotein produced and induced in monocytes and endothelial cells (EC), fibroblasts and macrophages. A comprehensive study by Faulk et al (20) used two types of specific antibodies for TF in an immunohistological study which localized TF in placental macrophages, EC or fibroblasts-like cells, but not in trophoblasts. A study by Lakasing et al (21) supported

this findings by localizing TF in perivascular cells in the placenta. However, other studies have demonstrated that placenta throphoblasts are able to synthesize and express TF. Increased levels of TF were demonstrated by activity assays and reactivity by western-blot in STB membranes isolated from villi (22-23). It has been demonstrated the presence of large amounts of ready-to-use TF in the placenta (24) . Teleologically, this seems to be essential for the maintenance of haemostasis in the placenta. The inhibitor of the TF activation pathway is TFPI; TFPI is produced and secreted by endothelial cells, potentially by microvascular EC (25). TFPI mRNA is highly expressed in placenta, mostly in megakaryocytes and endothelium of small vessels and macrophages in the villi of term placenta (26). During placental development, TFPI is expressed in STB and vascular endothelium from 10 weeks to term. The relative expression of TFPI mRNA was studied in several organs (23) and compared to other endothelium specific proteins: thrombomodulin (TM), von Willebrand factor (vWF), and TF. TFPI in placenta was found to be most abundant among the examined organs, while the level of vWF and TM were relatively low. Sprecher et al (27) have cloned the gene from the placenta and defined it as TFPI-2, which is homologous to TFPI/lipoprotein associated coagulation inhibitor. Although TFPI-2 has similarity in the domain structure and partial homology to TFPI, its inhibitory spectrum for serine protease is reported to be different from that of TFPI. TFPI-2 is a poor inhibitor of TF/VIIa complex, as compared to TFPI, but it is a strong inhibitor of factor Xa, plasmin and trypsin. TFPI-2 is found in the maternal serum of normal pregnancies , its serum level is increased with pregnancy progress, and it is relatively high in the sera of patients with trophoblastic disease. It was demonstrated that TFPI-2 is also expressed in human tissues, such as liver, skeletal muscle, heart, kidney

and pancreas (28). It can be speculated that potency of blood vessels is maintained through TFPI blocking of the endothelial initiated coagulation, while TFPI-2 having different affinities for coagulation complexes- may play a similar function in placental STB to protect blood flow in the villous spaces from hypercoagulation. Fibrinolysis is activated by plasminogen activators (PA) t-PA and urokinase (U-PA) and is inhibited by plasminogen activator inhibitor type 1 (PAI-1) or type 2. Both activators and inhibitors are secreted by vessel cells following stimulation by cytokines and growth factors (29). Two distinct fast acting inhibitors of PA have been identified. immunological analysis have shown that PAI-1 and PAI-2 are present in normal human placenta tissue (30) and that plasma levels of both inhibitors increase during the course of pregnancy (31) . PAI-1, the physiological inhibitors of both urokinase and t-PA, is synthesized mainly by endothelial cells, and is a primary regulator of the fibrinolytic system in vivo. Overexpression of PAI-1 may compromise normal fibrin clearance mechanism and promote pathological fibrin deposition, when the clotting cascade is activated. The presence of PAI-1 and expression of mRNA have been studied in normal and pathological pregnancies (29, 31). Plasma and placental PAI-1 levels were significantly elevated in pregnant women with severe PE. PAI-2, initially isolated from human placenta (32), is produced mainly by macrophages and keratinocytes, but evidence points to its presence and possible role in placental cell invasion in the uterus (33). Estelles et al (29) claimed that elevated plasma levels of PAI-1, but not PAI-2, have been implicated in mediating fibrin deposition and occlusive lesions, that occur within the placenta vasculature of villous tissue obtained from PE and IUGR. Limited information is available on factors regulating production of PAI-1 within either normal or PE/IUGR

placenta. A variety of factors, such as cytokines, growth factors and changed blood flow, are capable of affecting the expression of PAI-1, as well as that of TF. Kanfer et al have measured procoagulant and anticoagulant fibrinolytic and antifibrinolytic (TF, TM, t-PA urokinase, PAI-1 and PAI-2) activities in placenta extracts as well as the amount of fibrinoid deposition in term healthy placentas and in placentas from hypertensive PE women. (34). It was demonstrated that the hypertensive group had a higher placental content of PAI-2 and contained fibrinoid material. The increased documented change in PAI-2 could explain the imbalance of local fibrinolytic system in gestational complications. The presence of a normal placental vasculature is a fundamental feature of fetal development.

It is known that inherited (protein C, protein S, antithrombin deficiencies) or acquired (antiphospholipid antibodies) causes of thrombophilia can be associated with obstetric complications as early and late fetal losses. (35; 36). The thromboxane A2 (TXA2) has a pivotal role in the obstetric pathology, because is a powerful proaggregatory and smooth muscle cell constrictor agent (37) In some different districts two isoforms of its receptor (TP) are expressed, which have an antithetic function. TXA2 is platelet agonist, smooth muscle cell constrictor, and mitogen. Urinary TX metabolites (TX-M) excretion is increased in syndromes of platelet activation and early in both normal pregnancy and in pregnancy-induced hypertension. (38) A further increment occurs in patients presenting with severe preeclampsia, in whom TX-M correlates with other indices of disease severity. (37) TXA2 exerts its effects through a membrane receptor (TP, of which two isoforms (alpha and beta) have been cloned. Overexpression of TP in the vasculature under the control of pre-proendothelin-1 promoter (38) results in a murine model of intrauterine-

growth restriction (IUGR), which is rescued by timed suppression of TX synthesis with indomethacin. IUGR is commonly associated with maternal diabetes, maternal diabetes, or cigarette smoking,

conditions associated with increased TXA₂ biosynthesis. (37, 38, 39) Recently, in a murine model it has been demonstrated that the overexpression of the TP (genotype TP⁺⁺) is associated to a phenotype very similar to that of the human IUGR. Embryos from TP⁺⁺ females were consistently smaller in size and body weight compared to embryos from wild type females. Moreover, gross examination showed that the placentas of the TP⁺⁺ females were smaller than those of wild-type mice, but no thromboses were observed. Moreover, it is known that the overexpression of human TP in mice causes a phenotype very similar to that of human IUGR. (38).

During the last years, a great interest has been given to the haematological causes of recurrent miscarriage and of complicated pregnancy. It is well known that women affected by congenital or acquired thrombophilic alterations have a significant increase in miscarriage and complicated pregnancy rate (35).

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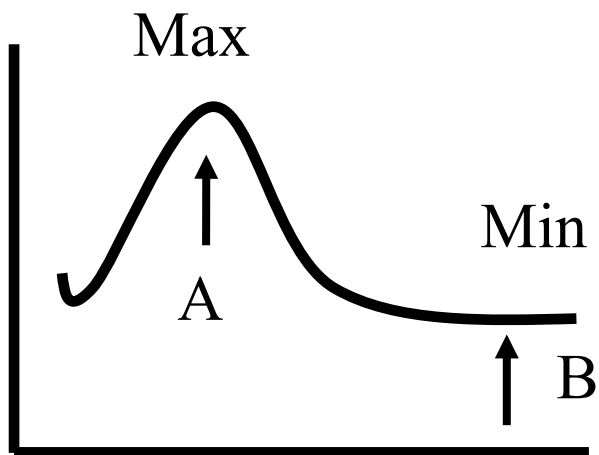
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DOPPLER and Pregnancy

Doppler studies are generally used to screen for IUGR or PE, with the possible exception of uterine artery Doppler. Rather, Doppler studies provide greater specificity after identifying a fetus with a suspected IUGR or fetal compromise. Although techniques such as Doppler velocimetry have improved the assessment of High risk fetuses, the challenge that has not been adequately addressed is how to identify those pregnancies that need to be referred for investigation and closer surveillance.

Blood flow in arterial vessels, mainly the umbilical artery, the middle cerebral artery (MCA) and the foetal thoracic descending aorta, can be assessed using various indices. These indices include the systolic (S) to diastolic (D) ratio, pulsatility index (PI) ($PI = \frac{S-D}{V_m}$, where V_m is the mean of maximal velocities throughout the cardiac cycle) or resistive index (RI) ($RI = \frac{S-D}{S}$).

The PI has the advantage of values even when the diastolic flow component is absent. Impedance to flow into the ductus venosus or into inferior vena cava can also be measured by calculating PI, referred to as PI for veins.



PULSATILITY INDEX = $A-B / \text{Mean}$

POURCELOT RATIO = $A-B / A$

SYSTOLIC/DIASTOLIC RATIO = A/B

Fig. 1: Illustration of commonly used indices of Doppler waveform

Uterine artery Doppler

Doppler US provides a non-invasive method for the study of the uteroplacental circulation. In normal pregnancy, impedance to flow in the uterine arteries decrease with gestation, which may be the consequence of trophoblastic invasion of the spiral arteries and their conversion into low-resistance vessels. PE and IUGR are associated with failure of trophoblastic invasion of spiral arteries and Doppler has then shown that impedance to flow in the uterine arteries is increased. Doppler sampling can be performed at the level of the main uterine arteries at the level of the arcuate arteries (1) or as a combined or as a combined approach of both sampling sites (2). Color Doppler visualization of the uterine arteries at the crossover with the external iliac artery is now the sampling site of choice. The normal waveform pattern shows a low resistance, high flow pattern throughout diastole (Fig. 2-3). The abnormal waveform patterns show high resistance and protodiastolic notch (Fig. 4-5).

Gestational age (GA) at sampling is usually 16-20 weeks and 22-24 weeks in a one-stage or two stage screening. High impedance to flow is generally assessed by RI or PI with cutoff values of 0,58-0,63 and 1,85, respectively, at these GA. Early diastolic notch when bilateral is another and more stringent way of describing abnormal results. Notch quantification has not proven to be useful. A series of screening studies involving assessment of impedance to flow in the uterine arteries have examined the potential value of Doppler in identifying pregnancies at risk of PE or other complication of impaired placentation. The results of an analysis of 15 studies (3) show that increased impedance to flow in the uterine arteries is associated with an increased risk of developing PE, IUGR, and perinatal death. In addition, women with normal impedance to flow in the

uterine arteries constitute a group at low risk of developing such impaired placentation related complications. Increased impedance to flow identified approximately 50% of pregnancies that will develop PE and around 30% of those that will develop IUGR subsequently. Abnormal Doppler is better at predicting severe rather than mild disease. The sensitivity for severe disease requiring premature delivery was approximately 80% for PE and 60% for IUGR. In the pooled data from all studies, the likelihood ratio for the subsequent delivery of a growth-restricted infant, occurrence of PE and perinatal death in women with an abnormal result was 3,7 5 and 2,4 respectively and the likelihood ratio was 0,7, 0,5 and 0,8 respectively for women with normal Doppler results. The incidence of complications varied from 2% to 24%; predictive values increased with the prevalence and the false positive rates decreased with gestation. Bilateral notches are found in 20-30% of pregnancies at 12 weeks, through to 10-20% at 16 weeks, around 10% at 20-22 weeks and less than 5% at 23-24 weeks of gestation. (4).

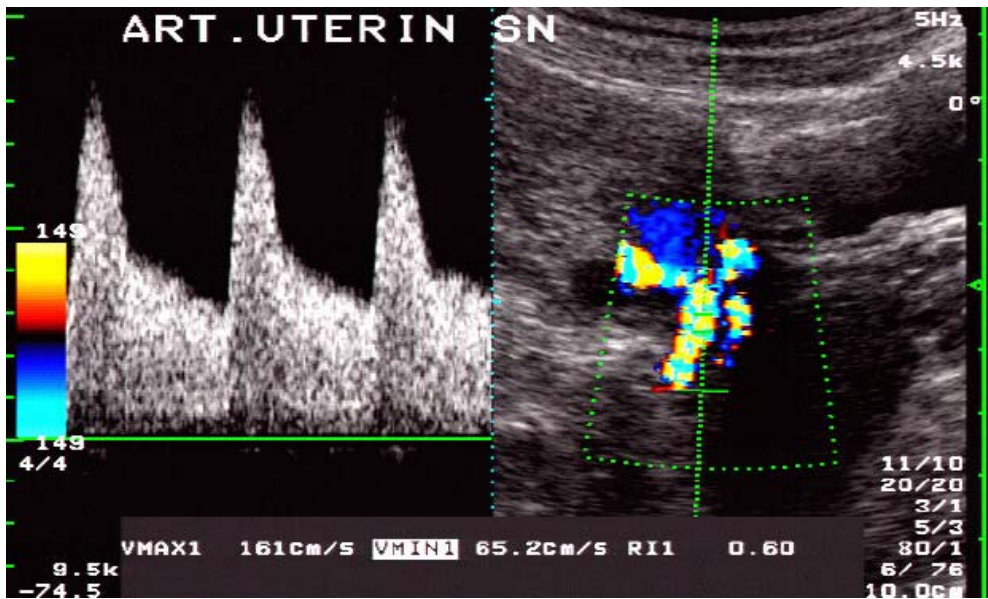


Fig. 2 Uterine left artery morphology, velocity waves and RI value were regular. The normal waveform pattern shows a low resistance, high flow pattern throughout diastole. Blood flow in left uterine artery in normal fetus.

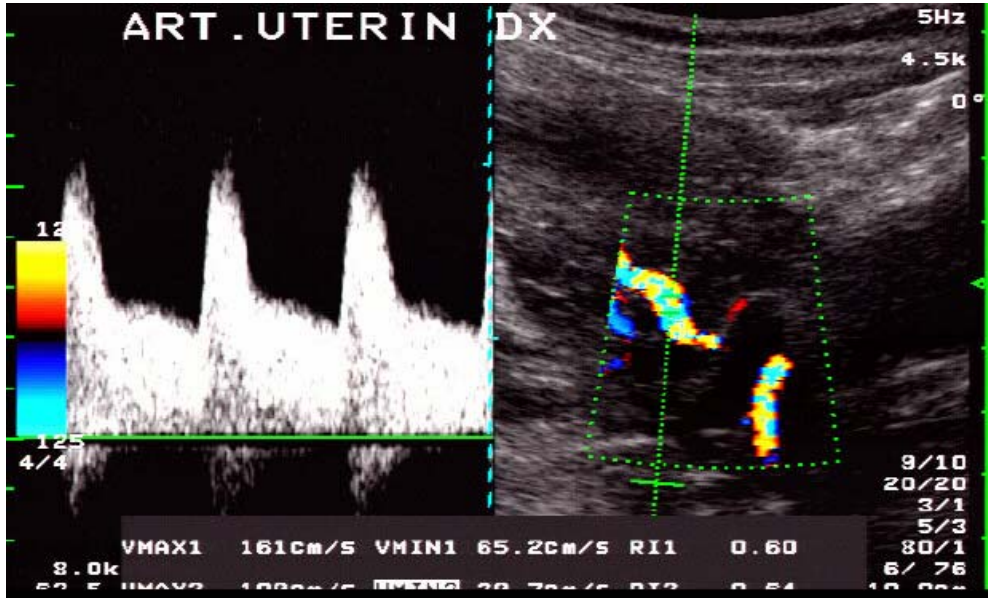


Fig. 3 Uterine right artery morphology, velocity waves and RI value were regular. The normal waveform pattern shows a low resistance, high flow pattern throughout diastole. Blood flow in left uterine artery in normal fetus.

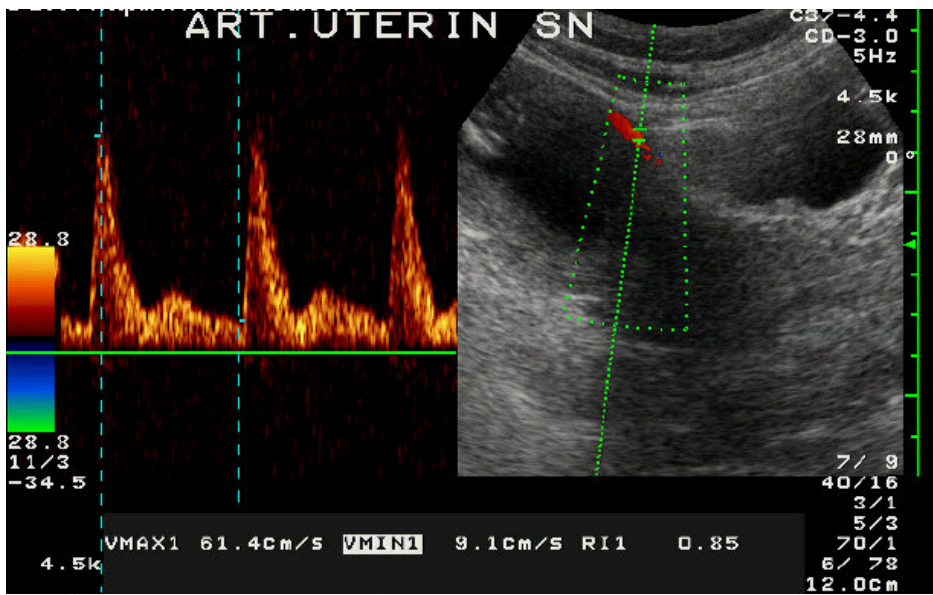


Fig. 4 Uterine left artery morphology, velocity waves and RI value were irregular. There is protodiastolic incisure. The abnormal waveform pattern show high resistance and protodiastolic notch. Blood flow in left uterine artery in fetus with intrauterine growth retardation (IUGR).

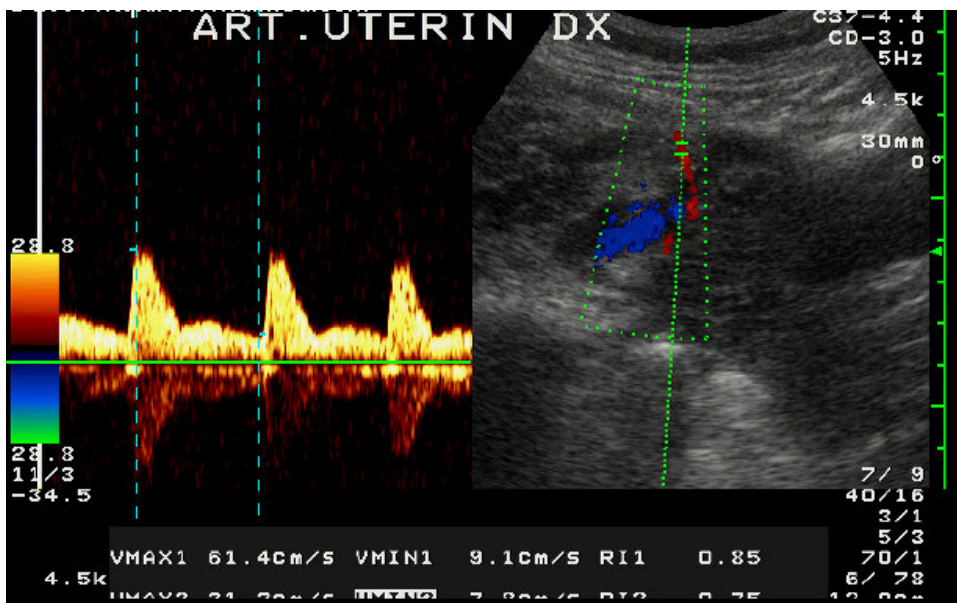


Fig. 5 Uterine right artery morphology, velocity waves and RI value were irregular. There is protodiastolic incisure. The abnormal waveform pattern show high resistance and protodiastolic notch. Blood flow in right uterine artery in fetus with intrauterine growth retardation (IUGR).

Umbilical artery Doppler

Decreased resistance to flow in the umbilical artery is normally seen between the fetal bladder and the placenta cord insertion and due to the reservoir effect of the placenta on impedance to flow along the umbilical cord. This will, however, remain within normal limits in normoxemic fetuses with normal placental resistance. (Fig. 6) The flow in the umbilical artery is a reflection of the placenta resistance mainly and, to a certain extent, of the fetal systemic resistance. However, when the diastolic component is reduced or absent (Fig. 7-8) or mainly reversed (Fig. 9) fetal cardiac decompensation is possible. Metanalyses suggest a 35-40% decrease in perinatal mortality when considering one umbilical Doppler measurement in high risk fetuses (5). This impressive effect of umbilical artery Doppler is mainly due to the high positive predictive value of absent/reversed and diastolic flow on perinatal mortality. (6) Absence and especially reversal of end diastolic flow of the umbilical artery Doppler is a worrisome finding associated with increased risk of perinatal and neonatal death as well as other complications. Elevation of umbilical artery Doppler resistance with diastolic flow is less predictive.

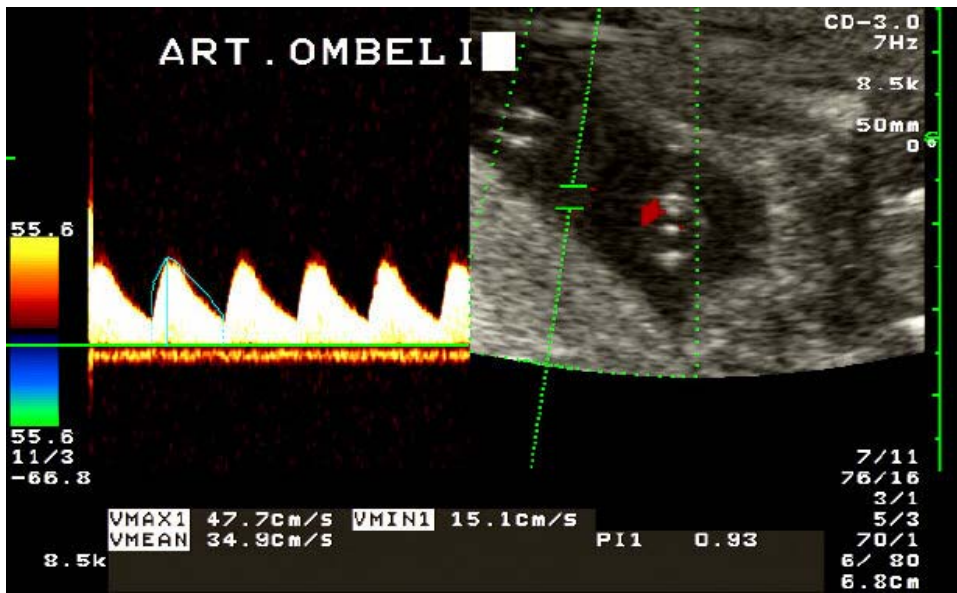


Fig. 6 Blood flow in umbilical artery (UA) in normal fetus. Umbilical artery morphology, velocity waves and PI value were regular.

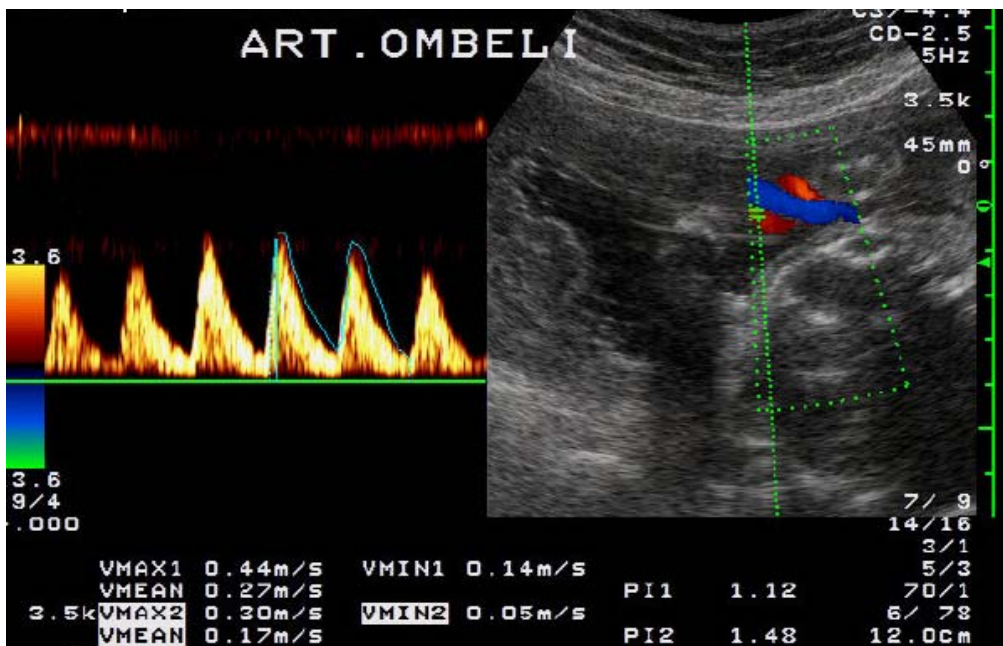


Fig. 7 Blood flow in umbilical artery (UA): the diastolic component is reduced.

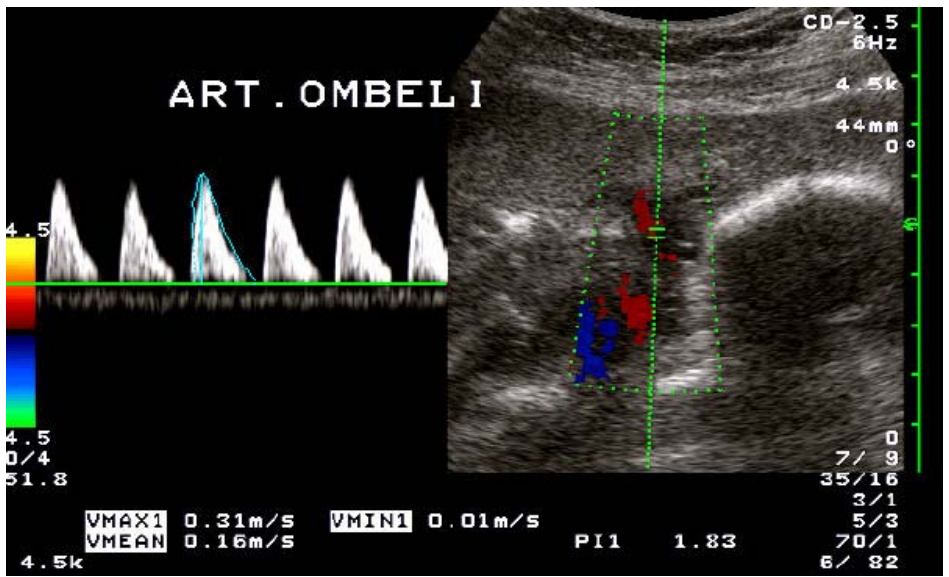


Fig. 8 Blood flow in umbilical artery (UA): the diastolic component is absent.

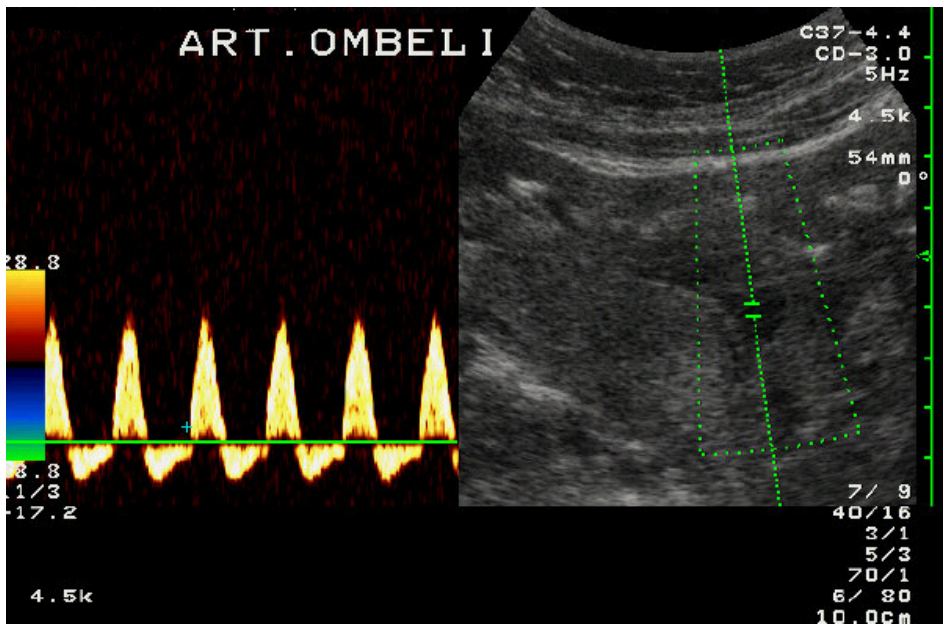


Fig. 9 The diastolic component is reversed. Umbilical artery morphology, velocity waves and PI value were irregular. There is reversal of end-diastolic flow in the umbilical artery (ARED flow).

Middle cerebral artery Doppler

The sampling site of Middle cerebral artery (MCA) Doppler is along the sphenoid wing at the internal third of the vessels. This vessel often presents with an ideal angle of insonation. Special attention must not press on the transducer, which could result in a significant decrease in diastolic flow in the MCA (7). PI in the MCA decrease with the gestation (8) Doppler easily shows vascular redistribution. This redistribution includes a decrease in resistance to flow in the MCA together with an increase in the resistance to flow in the fetal thoracic aorta. The resistance pattern of the middle cerebral artery Doppler should always be greater than the umbilical artery Doppler resistance before birth. (Fig.10) In the setting of fetal hypoxia, blood is preferentially shunted to the brain as the “brain sparing” adaptive response (Fig. 11). A middle cerebral artery Doppler waveform showing less resistance (lower S/D ratio PI or RI) than the umbilical artery Doppler is the reverse of the normal relationship and is diagnostic of the brain sparing effect. Sterne et al (9) found that this redistribution is associated with low birth weight and academia, and the severity of the abnormal Doppler pattern correlated with a shorter interval to delivery and the need for emergency delivery. The middle cerebral artery Doppler may be more sensitive than umbilical artery Doppler for identifying fetuses at risk. SGA fetuses with normal umbilical artery Doppler but abnormal uterine and MCA have an increased risk of developing distress and being delivered by emergency caesarean section. Furthermore, abnormal velocimetry of the uterine and MCA was independently correlated with the risk (10).

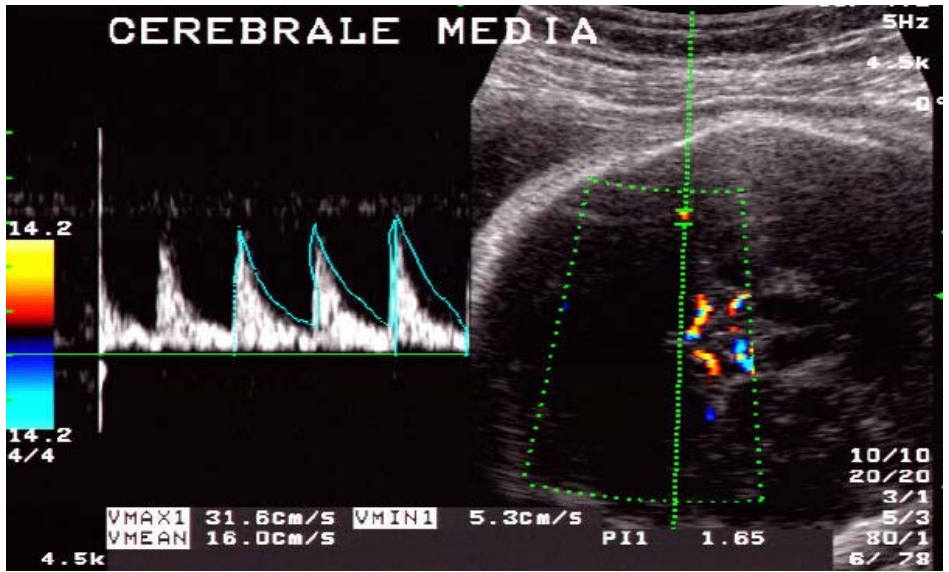


Fig. 10 Middle cerebral artery (MCA). The resistance pattern of the middle cerebral artery Doppler should always be greater than the umbilical artery Doppler resistance before birth.

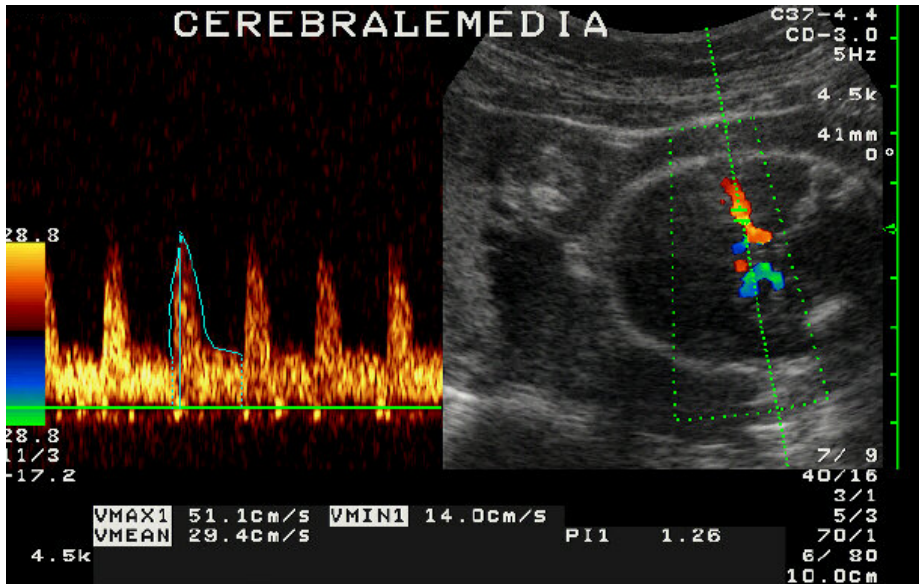


Fig. 11. A middle cerebral artery Doppler with the “brain sparing” adaptive response. The waveform showing less resistance (lower S/D ratio PI or RI) than the umbilical artery.

Venous Doppler

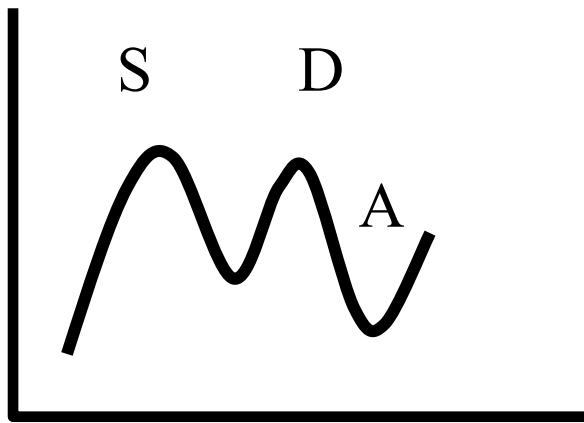
Doppler examination of the fetal veins is feasible and concerns mainly the ductus venosus and the inferior vena cava. The advantage of ductus venosus (DV) (Flow pattern Fig. 12) over the inferior vena cava in the assessment of IUGR fetuses is that the wave in the ductus venosus gets reversed only in severely compromised fetuses, thus acting as a sign of fetal demise. The DV can be identified with color flow imaging and duplex doppler (Fig. 13)

Imaging is performed either in sagittal or an oblique section of the fetal abdomen to visualize the communication between the umbilical vein (portal sinus) and the inferior vena cava. Presence of characteristic high velocity signal in the color-doppler mode ensures identification. Recording is taken in the inlet of the DV with an insonation angle as near to the long axis of the vessels as possible (11). Blood flow patterns may reflect change in pressure between the vessels and the right atrium throughout the cardiac cycle. Velocities are maximal during ventricular systole (S wave) in relation with a rapid filling of the atria; the second peak (D wave) corresponds to early ventricular diastole and flow velocity is minimal during atrial contraction (A wave). As with fetal arteries, various indices can be used to describe the waveform patterns of the DV. These indices include S/A ratio, the peak velocity index for veins (PVIV= A-A/D) and PIV (PIV= S-A/ time averaged maximum velocity).

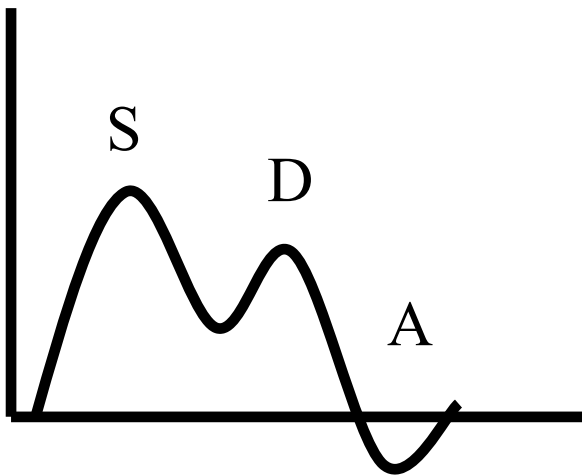
Various pathologic situations can influence the normal flow pattern (fig 14):

- a decrease in myocardial contractility, which can be seen in heart compression by pericardial or pleural effusion but also in subendocardial ischemia in severely acidotic fetuses;

- an increase in cardiac afterload which can be seen with high placental resistance or in pulmonary stenosis with intact septum;
- an increase in cardiac preload, which impairs complete auricular emptying seen in hypervolemia in a recipient in twin-to-twin transfusion syndrome and other situations which increased cardiac output.



A)



B)

Fig 12. Ductus venosus flow patterns. A: Normal waveform pattern. Normal systole consist of two peaks, with the first corresponding to ventricular systole (S) and the second to ventricular diastole (D). reduced velocity is observed during atrial diastole (A) but flow is always continuos above the baseline. B: Abnormal waveform pattern with reversal of A wave.

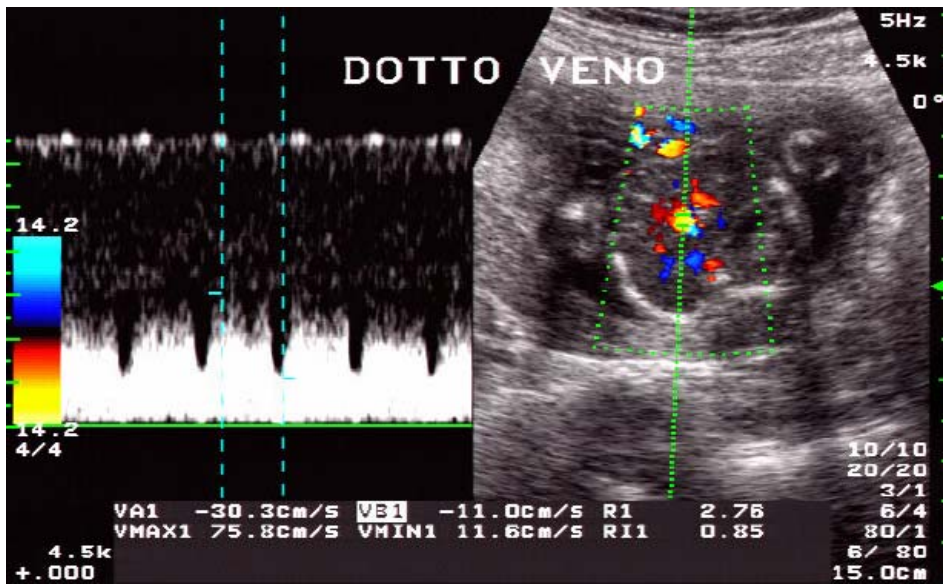


Fig. 13 Ductus venosus flow patterns. A: Normal waveform pattern. Normal systole consist of two peaks, with the first corresponding to ventricular systole (S) and the second to ventricular diastole (D). Reduced velocity is observed during atrial diastole (A) but flow is always continuos above the baseline.

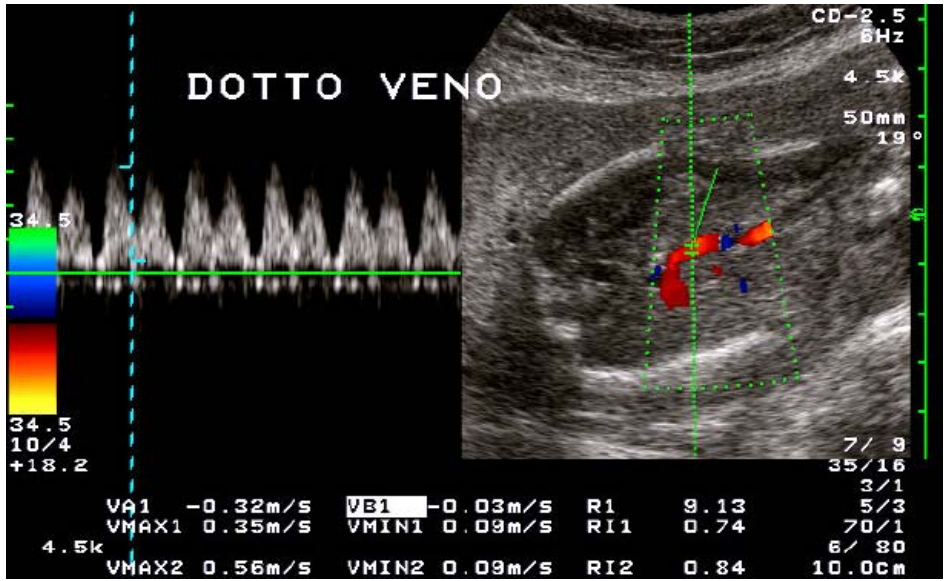


Fig. 14 Abnormal waveform pattern with reversal A wave.

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OBJECTIVES OF THE STUDY

In the first part of the study, we will enroll the number of patients expected to accomplish the aims of the study. We will select at least 15-30 pregnancies complicated for the presence of pre-eclampsia (the occurrence of gestational hypertension with significant proteinuria) and severe fetal growth restriction (FGR). During pregnancy women will undergo serial Doppler examination of the uterine and umbilical arteries. Women will undergo routine evaluation of uterine arteries and umbilical artery (UA). Color Doppler examination of target vessels will be performed according to Bilardo (Am J Obstet Gynecol 1990; 162: 115-20) and Albaiges (Obstet Gynecol 2000; 96: 559-64). Doppler measurements will be taken from the frozen image after at least five consecutive uniform flow velocity waveforms during period of fetal rest and apnea. Abnormal UA Doppler flow velocimetry will be defined as a pulsatility index (PI) >2 standard deviations (SD) above the mean for gestational age by local reference values and/or absence or reversal of end-diastolic velocities. Fetuses with abnormal UA Doppler PI will be longitudinally evaluated by serial fetal Doppler examination of ductus venosus (DV) flow velocimetry, by non-stress test and biophysical profile score. Uterine artery flow velocimetry will be considered abnormal in cases of bilateral uterine artery notches or when the mean PI of the two vessels will be $>95^{\text{th}}$ centile of the reference range for gestational age in our study population. For DV, an elevation of the peak velocity index $>2\text{SD}$ above the gestational mean age will be considered abnormal. The time interval between Doppler examinations will depend on Doppler investigation results. In case of UA absent or revers end-diastolic flow, fetal monitoring will be performed daily. Moreover, fetal growth will be monitored. We will pay particular attention to select placentas from pregnancies with newborns

showing a percentile below 3th or 5th centile. Using this cut-off, a more severe setting will be enrolled and it is conceivable that a greater effect on the gene expression of variables investigated will be identified.

During the *first phase* of the study we will investigate whether different areas of placenta express different amounts of the same marker in order to obtain information about homogeneity of expression.

During the *second phase* of the study, we will evaluate the expression of markers considered in pathological placentas as compared to normal ones. In particular, our Unit will obtain slices from all collected placentas, then we will obtain homogenate from each section. Finally, we will extract total RNA and synthesise cDNA determining the expression of each marker in all cDNA samples obtained.

During the second phase of the present application, all scheduled laboratory variables will be determined in all patients enrolled. We will obtain specific complementary DNAs from placentas of women with complicated pregnancies. Then we will investigate allele and genotype frequencies of common gene variations. In parallel, in placenta obtained in women presenting with a complicated pregnancy, the modulation of the gene expression of a series of factors of the coagulation/fibrinolytic pathway will be investigated.

We will isolate total RNA by placentas after homogenization with 1 ml Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Phenol-phase separation will be performed with gel-phase tubes (Eppendorf, Hamburg, Germany) in order to prevent protein contamination of the RNA. RNA will be dissolved in 20 µl RNase-free H₂O and exposed to 55 °C for 5 min to increase solubility. The RNA

concentration will be determined at 260 nm with a plate-reading spectrophotometer (SPECTRAMax™; Molecular Devices, Wokingham, UK).

Then, specific cDNAs will be synthesized. Briefly, a mixture of 0.5 ug total RNA and 0.25 ug Oligo dT (Invitrogen) per sample will be subjected to 65 °C for 5 min to promote primer annealing. A volume of 8 ul RT master mix containing 1× RT buffer, 25 mM dTT, 1.25 mM dNTP and 200 U moloney murine leukemia virus reverse transcriptase will be added, and the mixture incubated at 37 °C for 70 min. The RT reaction will be terminated at 95 °C for 5 min, whereupon samples will be frozen to -20 °C.

Genomic DNA will be extracted from all probands enrolled. DNA will be extracted from white blood cells using the salting-out method. All gene variants will be investigated by using PCR and restriction enzymes. Primer will be "ad hoc"; designed and synthesized. Random DNA manual or automated sequences will be made to verify results obtained during the routine screening of genetic polymorphisms investigated. An independent and blinded control of clinical evaluation of patients, including all information obtained, and of laboratory data will be done by investigators who participate to the present project.

PCRs will be carried out in 50 microl samples, using a Perkin Elmer-Cetus thermal cycler (Perkin-Elmer, Norwalk, CN), containing 0,1 microg of genomic DNA, 10 pmol of each primer, 125 microM of dNTP, 5 mM of Tris HCl pH 8.3, 50 mM of KCl, 2.5 mM of MgCl₂, 0.01% (peso/volume) of gelatin, and 1 U of Taq polimerase. The solution will be overlaid with 50 microl of mineral oil and after a denaturation at 95 °C for 3 min, will undergo 30 cycles of amplification, 1 min at 95 °C, 1 min at 56-60 °C, and 2 min at 72 °C. Then, 5 microl of the amplification product will undergo agarose gel electrophoresis in TAE buffer (40 mM TRIS-Acetato, 1 mM EDTA pH 7.7) containing 0.5microg/ml of

ethidium bromide, and visualized under UV. Genotyping analyses will be carried out according to standard procedures. Random DNA manual or automated sequences will be made to verify results obtained during the routine screening of genetic polymorphisms investigated. An independent and blinded control of clinical evaluation of patients, including all information obtained, and of laboratory data will be done by investigators who participate to the present project. SNPs data will be confirmed by direct fluorescent dye-terminator cycle sequencing of PCR products and subsequent analysis on capillary-based autosequencers (models ABI-3100 and ABI-310, Applied Biosystems).

From placenta sampled by other Units participating into the current study and stored at -80°C, RNA will be purified using standard methods e cDNA will be obtained. Finally, the material will be tested using the quantitative PCR technology for the placental expression profile of a series of genes, i.e. tromboxane A2 receptor, PAI-2, tissue factor, TFPI, etc. Constitutive expressed genes, such as beta-actin and GAPDH will serve as standard for the normalization of the results.

The above-mentioned activity will be also performed for other Centers that collaborate to the investigation. Each transcript will be studied taking advantage on specific probes. Briefly, total RNA will be isolated from tissue using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Phenol-phase separation will be performed with gel-phase tube (Eppendorf) in order to prevent protein contamination of the RNA. Then, RNA will be dissolved in 20microlters RNase-free water and exposed to 55 °C for 5 minues to increase solubility. The RNA concentration will be detrmind at 260nm with a plate-reading spectrophotometer. For the cDNA synthesis , amixture of 0.5 micrograms of total RNA and 0.25 micrograms Oligo dT (Invitrogen) per sample will be

subjected to 65°C for 5 min to promote primer annealing. A volume of 20 microliters of Rt reaction containing 8 microliters of RT master mix containing 1X RT buffer, 25 mM dTT, 1.25 mM dNTP and 200 U of moloney murine leukemia virus reverse transcriptase will be incubated at 37°C for 70 min. The probes that will be used for quantification of targets and endogenous controls will be designed according to the TaqMan technology (Applied Biosystems) using the Primer Express (Applied Biosystems) computer software. The real-time PCR will be performed on the ABI prism 7700 (Applied Biosystems) PCR and detection instruments. The fluorescent signal from the dye 6-carboxyfluorescein (6-FAM) at the 5' end of the probe will be quenched by another fluorochrome, TAMRA, at the 3' end. The quenching effect will terminate as the probe is cleaved due to the 5' exonuclease activity of the Amplitaq Gold (Applied Biosystems) enzyme and a fluorescent signal emitted. The emittance will be proportional to the amount of amplified product, until it reaches the lag phase of the PCR. A threshold value setted above the baseline will reflecte by the average change of emittance during the first PCR cycles. The CT value (cycle number), is the point at which the emittance passes above the baseline and thus mirrors the accumulation of PCR product in a specific well of the PCR reaction. The real-time PCR reactions will be performed on plates using adhesive seals as covers. A master mix will be prepared for each plate and each quantitation target in 25 microliters reactions, with final concentrations of 1x Buffer A, 3 mM MgCl₂, 0.2 mM dNTP, 200 nM probe and primers and 1.25 U Amplitaq Gold. The PCR will be programmed as follows: initial denaturation at 95 °C 10 min followed 40 cycles of 95°C for 20 sec and 58-60°C for 20 sec . Gene polymorphism of hemostatic factors will be studied assessing factor V Leiden, G20210A prothrombin, C677T

methylenetetrahydrofolate reductase (MTHFR), plasminogen activator inhibitor 4G/5G, and platelet glycoprotein PL A1A2 gene mutations, and dosing hcy, proteins C, S, free S, antithrombin III, anticardiolipin antibodies IgG and IgM, dilute Russel's viper venom time, activated partial thromboplastin time, Factor VIII, Factor XI, lipoprotein (Lp)(a), and plasminogen activator inhibitor activity (PAI-Fx). During the course of the second phase of the present application, the accomplishment of all laboratory evaluations scheduled will provide a complete output of variables investigated in different settings of patients and in the related groups of controls. This constitutes one of the major goal of the present application: the setting up of an archive that will include all clinical information obtained and DNA samples. This archive may be of help for further clinical studies addressing this issue and looking for new strategies for the prevention, diagnosis, and therapy of some complicated pregnancies. During the third phase of the present application, the statistical analysis of all data obtained will be carried out looking for significant associations.

All the statistical analyses will be performed according to the Statistical Package for Social Science (SPSS 10.0 for PC). The significance of any difference in means will be evaluated by non-parametric test, whereas the significance of any difference in proportions was tested by chi-square statistics. The allele frequencies were estimated by gene counting, and genotypes were scored. The observed numbers of different genotypes will be compared with those expected for a population in Hardy-Weinberg equilibrium using a chi-square test. The significance of the difference of observed alleles and genotypes between the groups will be tested using the chi-square analysis also after grouping homozygous and heterozygous carriers of each allele, investigating in addition

to a codominant model the possibility of a relationship assuming a dominant or recessive model. Statistical significance was taken as $p < 0.05$. The statistical analysis will calculate exposure Odds Ratios (ORs) and 95%-confidence intervals (CI) as estimates of the relative risk associated with specific risk factors: i. e., gene variants, environmental factors, and their combination. ORs will be calculated by cross-tabulation and unconditional logistic regression. Statistical uncertainty will be taken into account by the construction of confidence intervals based on a Poisson distribution of the number of events, i. e., by the method of Woolf or according to the standard error derived from the maximum likelihood estimator of the model. Since incident cases and population controls are included, the ORs represent measures of the incidence rate ratio. Adjustment for putative confounders will be performed by multivariate modelling.

The joint effect of risk factors, either both genetic (gene-gene interaction) or genetic and acquired (gene-environment interaction) will be assessed. Basically, risk estimates will be derived for main effects and joint effects. Interaction will be defined as the risk for the joint presence exceeding the sum of the separate effects (testing for departure from additivity). This will be done by logistic regression, with multiplicative-additive models. The sample size will be sufficient to detect moderate departures from additivity for genetic variants which are still not multiplicative, and to detect small departures from additivity for the gene-environmental combination of risk factors - since these are more common. During the course of the third phase of the present application, it is planned to get results of the statistical analysis. All information obtained will serve to better define the clinical and genetic profile of clinical settings enrolled.

During the fourth phase of the present application, information deriving from the statistical analysis will be evaluated. Results obtained will be matched with data from clinical studies of the literature. At the end, all data obtained will serve as starting point to set up new strategies for the primary and secondary prevention of pregnancy complication investigated. During the course of the fourth phase of the present application, it is planned to set up new strategies for the primary and/or secondary prevention of pregnancy complication investigated.

One of the most important intermediate results will be the accomplishment of a sample size comparable to that scheduled in the aims of the present applications. All clinical and laboratory information obtained will provide an illustration of patients and controls enrolled. We suggest that the final step of the project would be positively completed whether we will obtain the accomplishment of the statistical analysis of all data obtained in the entire group of women enlisted. This information will be presented in national and international meetings and will be submitted as original manuscripts to peer-reviewed international journals, quoted in Index Medicus. From a scientific point of view, the communication of results obtained in the course of the present application will allow to verify the meaning and the weight of our thoughts and conclusions of the study. Moreover, at the end of the study it will be possible to contact other Researchers in the field who, in addition to critically discuss conclusions obtained, will be able to verify in different and independent clinical settings of patients and controls the validity of clinical protocols of screening and prevention provided by the present application.

We expect that the present study will provide results that will allow us: (1) to set up new protocols for the primary identification of at risk subjects; (2) to arrange new therapies on

the basis of the knowledges derived from this kind of study; (3) to identify a genetic risk profile that will account of interactions with environmental, acquired, and inherited risk factors; (4) to set up an archive that will include all clinical information obtained and DNA samples. This archive may be of help for further clinical studies addressing this issue and looking for new strategies for the prevention, diagnosis, and therapy of complicated pregnancies. If we will be able to attain prefixed aims, in addition to the improvement of our knowledge about the physiopathology, we will obtain to the woman, his family, and the whole community, a significant reduction of social and economic problems deriving from the occurrence of unsuccessful pregnancies.

To investigate whether different areas of placenta express different amounts of the same marker in order to obtain information about homogeneity of expression we start our study presenting data obtained from placentas of women with successful pregnancy outcome.

App. 1

Few studies have been carried out to investigate whether different areas of at term placenta express different amounts of markers involved in the placental haemostasis and angiogenesis. We evaluate correlation between factors involved in the local haemostasis and angiogenesis in term human placenta.

App. 2

After we investigate placenta expression profile of different coagulation markers in human placentas from complicated pregnancies.

App. 3

App.4

App. 1

EXPRESSION PROFILE OF DIFFERENT COAGULATION MARKERS IN HUMAN PLACENTAS FROM UNEVENTFUL PREGNANCIES

INTRODUCTION

The architecture and functions of the placenta present haemostatic problems, mainly the risk of haemorrhage. Placental trophoblasts express and produce coagulation components, participating not only in hemostasis, but also in placental vascular development and differentiation. The expression of tissue factor (TF), membrane phosphatidylserine and fibrin render the trophoblasts pro-coagulant, thus compromising the risk of bleeding while exposing the placenta to pro-thrombotic risk. Local inhibitory mechanisms act via TFPI and TFPI-2, thrombomodulin, annexin V. Pregnancy complications have been associated with abnormalities in the functions of these inhibitors. Moreover, different isoforms of the thromboxane A₂ receptor seem to be differently expressed in mice with fetal growth restricted fetuses. The aim of our study was to investigate whether different areas of placenta express different amounts of the same marker in order to obtain information about homogeneity of expression.

METHODS

Here we present data obtained from 4 placentas of women with successful pregnancy outcome. The placentas were dissected starting from the insertion of umbilical cord towards the peripheral zone. From each section RNA was extracted by means Invitrogen Trizol, Reagent. Then cDNA was obtained by means RT-PCR (Promega Reverse

transcription system), and the expression of TF, TFPI, TFPI-2, PAI-2, and thromboxane receptor α -isoform and β -isoform was evaluated in different areas of placenta by means of APPLERA ABI PRISM 7700. Data were expressed as percent of GAPDH for each sample analyzed.

RESULT

No significant difference ($p>0,05$) between central and peripheral areas of placentas was observed for all markers analysed. Data are shown in the Table.

CONCLUSION

The aim of our study was to investigate whether different areas of placenta express different amounts of the same marker in order to obtain information about homogeneity of expression. These preliminary data will be useful for comparing the expression of these markers in normal placentas with that of placentas from complicated pregnancies (preeclampsia, IUGR)

	TF%		TFPI%		TFPI-2%		PAI-2		TXA2R α		TXA2R β	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N	105	8,2	112,5	5,8	135	15,1	92	5,8	81	3,7	75	3,1
A	105,3	25,6	114	2,6	136	4,7	93	1,8	86,4	4,4	76	3,6
B	105,6	4,2	112,5	2,9	134	7,6	94,5	3,1	83,3	4	79	2,6

TABLE .1 Legend N: zone corresponding to the inserction of umbilical cord; A: zone next to N, B: marginal zone of placenta.

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App.2

Correlation between factors involved in the local haemostasis and angiogenesis in term human placenta.

ABSTRACT

Introduction: Few studies have been carried out to investigate whether different areas of at term placenta express different amounts of markers involved in the placental haemostasis and angiogenesis. A possible relationship between the expression of genes involved in the haemostasis and angiogenesis of human placenta has not been investigated.

Materials and Methods: Twenty-eight fresh human placentas (35-41 weeks of gestation) from uneventful pregnancies were dissected with two different methods. Quantity mRNA expression of TF, TFPI, TFPI-2, PAI-2, Anx V, VEGF, TM genes were evaluated by quantitative real time PCR system. Histology of each sample was graded .

Results: Gene expression of all the considered markers was not significantly different (ANOVA test, p always >0.05) in each area, using both the different methods of dissection. A significant correlation ($p<0.05$) was found between the expression of TF and TFPI-2. TF and TFPI-2 were significantly ($p<0.05$) associated with VEGF, whereas a stronger association ($p<0.01$) was found between TFPI and TFPI-2. TFPI and TFPI-2 were strongly associated with PAI-2 expression ($p<0.01$).

Conclusions: In placentas with central cord insertion, gene expression is not dependent on the method of sampling site. A significant relationship between haemostasis and angiogenesis in at term placentas was shown.

Key-words: placenta, TF, TFPI/TFPI-2, PAI-2, VEGF

Abbreviations: Tissue factor (TF); Tissue Factor Pathway Inhibitor (TFPI); Tissue Factor Pathway Inhibitor-2 (TFPI-2); type-2 Inhibitor of Plasminogen Activator (PAI-2); Annexin V (Anx V); Thombomodulin (TM); Vascular Endothelial Growth Factor (VEGF).

INTRODUCTION

In some gestational disorders, pre-eclampsia and some forms of intrauterine growth restriction (IUGR), trophoblasts fail to express most of the endothelial markers Zhou 1997, suggesting that this adhering phenotype switch is required for successful endovascular invasion and normal placentation (Fisher 1999). During pregnancy, the placenta is a putative source of tissue factor (TF), which is essential for embryogenesis (Erlich,1999), angiogenesis (Abe K, 1999), implantation and hemostasis (Parry 2000). The pro-coagulant tendency may render the placenta more susceptible to thrombotic risks, and excess activation of coagulation requires inhibitory mechanisms to avoid vascular occlusion (Aharon 2004).

The inhibitor of the TF action pathway , tissue factor pathway inhibitor (TFPI) is highly expressed in placenta (Aharon 2004) and inhibits factor Xa and factor VIIa-TF complex. TFPI is produced and secreted by endothelial cells. TFPI is expressed in placenta, mostly in megacaryocytes, endothelium of small vessels and macrophages (Edstrom CS 2000) TFPI-2 has been cloned from placenta and is homologous to TFPI/lipoprotein-associated

coagulation inhibitor. TFPI-2 is a poor inhibitor of the TF/factor VIIa complex, as compared to TFPI, whereas is a strong inhibitor of factor Xa. TFPI-2 mRNA is widely expressed in various adult human tissue and especially abundant in placenta. In the human placenta thrombomodulin (TM) is located on the endothelium of placental vessel and also on the capital membrane of syncytiotrophoblast (Maruyama I 1985): a physiological role of TM and TF in the maintenance of maternal blood fluidity inside the intervillous space has been suggested.

The type-2 inhibitor of plasminogen activator (PAI-2), a member of the serpin gene family is a fast-acting inhibitor of tissue-type and is expressed in trophoblast, monocytes, endothelial cells [Jensen, 1993]. It is known that it decreases in syncytiotrophoblast cells from IUGR placentas without or with preeclampsia.

Annexin V (Anx V), a calcium-dependent anionic phospholipid-binding protein localised in many tissues, is abundant in the placenta, but low concentrations are present in blood. Anx V has a high affinity for anionic phospholipids, which binds in a Ca^{2+} dependent manner, inhibiting procoagulant reactions requiring anionic phospholipid. It is abundant on trophoblasts and plays a pivotal role in the occurrence of fetal loss in a murine model [Wang X 1999]. Vascular endothelial growth factor (VEGF) and its receptors have been localized in the placenta during early pregnancy at the time when the syncytiotrophoblast penetrates maternal tissues before vascular development (Wulff 2002). VEGF expression was localized in the trophoblast, suggesting that endothelial cell differentiation, migration and proliferation are essential steps for building the primary vascular network (Vuorela 1999) During pregnancies complicated by preeclampsia and HELLP (Hemolysis,

Elevated Liver enzymes, and Low Platelets) syndrome, in which trophoblast invasion is disturbed, levels of VEGF are decreased (Zhou 2002).

Placental architecture and blood flow are not uniform across the chorioallantoic human placental disk. Proximity to the umbilical cord, basal plate or chorionic plate may influence perfusion. Furthermore, syncytial knots and villous fibrin are more common near the chorionic surface as well as near the placental margin compared to other locations in the placenta.

Although some studies investigated the expression of some genes involved in the occurrence of preeclampsia or IUGR (McCarthy C 2007, Roh 2005), to date few studies have been carried out to investigate whether different areas of at term placenta from uneventful pregnancies express different amounts of the above-mentioned markers in order to obtain information about homogeneity of expression. It has been postulated [Wyatt 2005] that if proximity to the cord was a key determinant of gene expression level, samples obtained near the marginally inserted cord would have lower expression than those obtained near the placental centre or from the margin opposite from the cord insertion.

Aims of our study were: to evaluate the expression of each factor in different sections of at term placenta and to investigate a possible relationship between the expression of different genes.

Materials and methods

Placental tissue acquisition

Twenty-eight fresh human placentas (35-41 weeks of gestation) from uneventful pregnancies were obtained immediately following uneventful spontaneous vaginal deliveries. Placentas were dissected in two spectral sections, the first was stored at -80 °C and then lyophilized, the second one was fixed in formalin 10%. In a first group of placentas (n=4), nine cubic sections (2x2 x all thickness cm), starting from the area closest to the cord insertion and proceeding toward the marginal area (Fig. 1) were obtained and stored at -80 °C. In a second group of placentas (n=8) 3 disks were obtained: chorionic surface, middle surface and basal surface. From each disk 3 samples (2x2x1 cm) were dissected starting from the cord insertion to lateral edge (Fig. 2). Two spectral pieces were cut from each section, one was stored at -80 °C and one was fixed in formalin 10%. A section from the chorionic to basal surface, was cut from other 17 placentas and stored at -80°C. Umbilical cord insertion was defined as central if located within the third of the placental disk radius. Placentas with marginal or velamentous cord insertion were excluded. RNA purification, reverse transcription and quantitative PCR were found. The frozen samples of placentas were lyophilized and total RNA was extracted by means of Invitrogen Trizol, Reagent (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's instructions. RNA was treated with DNase (2 U /□g of RNA), incubated in Gene Amp PCR System 2400 Thermal Cycler (Applied Biosystems, Warrington, UK) for 20 minutes at 37 °C, 3 minutes at 85 °C, 10 minutes at 4 °C. Electrophoresis on a 1% agarose gel was performed to confirm the presence and

quality of RNA. RNA concentration and purity were determined by UV absorption at 260 nm and 280 nm. Complementary DNA (cDNA) was prepared from the total RNA by means RT-PCR (Promega Reverse transcription system) (Promega, Madison, Wisconsin, U.S.A.). Quantity mRNA expression of TF, TFPI, TFPI-2, PAI-2, Anx V, VEGF, TM genes were evaluated by ABI 7700TM quantitative real time PCR system (Applied Biosystems, Warrington, UK) and compared to the human housekeeping glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) gene. Primer and probe sequences were either supplied as a ready mix, TaqMan® Gene Expression Assay (VIC-MGB for human GAPDH and FAM-MGB for human TF, human TFPI, human TFPI-2, human PAI-2, human Anx V, human VEGF, human TM). Each sample contained: 3 µl (0.04 µg/µl) of cDNA, Taqman Universal PCR Master Mix (12.5 µl), GAPDH primers and probe mix 20x (0.625 µl), target primers and probe mix 20x (1.25 µl) and RNase free water to a volume of 25 µl. Amplification was performed for 10 min at 95 °C, 45 cycles of 15 seconds at 95°C, 60 seconds at 60 °C. Gene expression levels were calculated using standard curves generated by serial dilutions of cDNA. A strong correlation between PCR efficiency of the internal control (GAPDH) and the target allowed the use of the ΔC_t -method to quantify comparable mRNA levels. Three independent analyses were performed with replicates.

The relative gene expression values were calculated by means of the comparative Ct method, using this arithmetic formula. $X_n = K(1+E)^{-\Delta C_t}$, where X_n = number of target molecules at cycle n; K= constant; E= efficiency of target assumed equal to efficiency of GAPDH; $\Delta C_t = C_{t,XX} - C_{t,R}$, where $C_{t,XX}$ = threshold cycle for target amplification, $C_{t,R}$ =

threshold cycle for GAPDH amplification. For amplicons designed and optimized according to PE Applied Biosystems guidelines (amplicon size < 150 bp), the efficiency is close to one. Assuming $K=1$, the amount of target, normalized to GAPDH is given by $2^{-\Delta Ct}$ [Lyvak 2001].

Histological analysis

Placental formalin- fixed paraffin-embedded sections (4 μm) were deparaffinized, rehydrated and stained with hematoxylin and eosin. The histology of each sample was graded based on Wyatt parameters: (a) villous size (large, medium or small) (b) amount of fibrin deposits (sporadic, medium or increased) and (c) prevalence of syncytial knots (sparse, intermediate or common). Then, the slides were classified into grade I-III, with grade I characterized by larger villi, sporadic fibrin deposits and sparse syncytial knots, whereas grade III characterized by small villi, increased amounts of fibrin deposits and common syncytial knots. Grade II was used for those samples that did not fulfill the criteria for either grade I or grade III. (Wyatt 2005).

Immunohistochemistry

Placental formalin- fixed paraffin-embedded sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with Peroxidase Block (0.03% hydrogen peroxide containing sodium azide) (DakoCytomation) for 5 minutes at room temperature, followed by brief rinsing in distilled water. The sections were blocked for 10 minutes at room temperature with a solution of 5 % normal goat serum, briefly rinsing in distilled water and then washed in TBS (DakoCytomation). Primary antibody for TF (American Diagnostica) 1:500 dilution was applied to cover specimen for one hour

followed by secondary antibody, peroxidase labelled polymer (DakoCytomation) for 30 minutes. Enhanced liquid DAB+ substrate-chromogen solution (DakoCytomation) was then applied to cover the specimen for 5 minutes. Then nuclei were displayed by means hematoxylin. Chromogen substrate solution created an intense color deposit (brown) around the antigen in the sample. The sections were visualized by microscope.

Statistical Analysis

Statistical analysis was performed by means of SPSS. All the results are presented as mean \pm SD . Differences between groups were analyzed by means of Mann-Whitney method. ANOVA was used to compare three or more independent samples. Correlation between parameters was assessed using the Spearman rank test. Values of $p < 0.05$ were considered significant.

Results

Gene expression of all the considered markers (TF, TFPI, TFPI-2 PAI-2, Anx V, VEGF, TM) was not significantly different (ANOVA test, p always ≥ 0.05) in the area closest to the cord insertion site compared to each of the peripheral ones , according to the method of dissection shown in Figure 1 (Tab 1).

Histological analysis showed, as expected, [Wyatt 201] grade I most prevalent at site 1A, which represents the medio-basal region. Histological changes for grade I are large villi, sporadic fibrin deposits and sparse syncytial knots. Grade III , as expected, was the most prevalent at site 3C, which represents the lateral-chorionic region. Histological features at this side included small villi, prevalent fibrin deposits and frequent syncytial knots.

We hypothesized that another method of sampling sections could show a difference in the expression of these markers, according to the findings of histological analysis. Thus, placental samples were obtained as shown in Figure 2.

Gene expression of chorionic, maternal and intermediate areas was performed in all 9 samples depicted in Figure 2. Each sample did not show a significantly different expression of all the markers (Table 3, ANOVA test, p always ≥ 0.05), as compared to any other sample.

Thus, we investigated a possible relationship between these markers in sections closest to the insertion cord of 20 normal placentas.

As showed in Figure 3, a significant correlation ($p < 0.05$) was found between the expression of TF and its inhibitor TFPI-2, mainly produced by placenta. In addition, TF and TFPI-2 were both significantly ($p < 0.05$) associated with a marker of angiogenesis, VEGF, whereas a stronger association ($p < 0.01$) was found between TFPI and TFPI-2. Both pathways inhibitors, TFPI and TFPI-2, were strongly associated with PAI-2 expression ($p < 0.01$).

Discussion

Although many authors have investigated human at term placenta, few data are available about a possible different expression of markers, depending on the sampling site. To date, it is not clear whether studies of gene expression have to be performed using a certain method for dissection of placenta.

In order to better define how to obtain placenta samples for following studies aimed at comparison of placentas from uneventful and complicated pregnancies, we first decided

to compare two different methods of dissection. In addition, we would like to verify by recruiting a relatively high number of normal placentas, the biological variability in the gene expression. Two different methods for dissecting placenta were used and, although histology showed, as in previous similar reports, [Wyatt 2001] the presence of different grades within normal placenta, no significant differences were observed in the gene expression. Wyatt and coll recently showed that the gene expression of VEGF was enhanced in the subchorionic lateral border compared to medial basal site.

At variance with Wyatt and coll, we first lyophilized the samples and furthermore used only placentas with central cord insertion. This procedure allowed us to obtain sections measuring exactly 2x2x1 cm. This could explain our different results.

Many studies have been reported in literature about TF/TFPI expression in placentas from pregnancies complicated by IUGR or preeclampsia, although it is not clearly stated usually which sections of placentas have been used. Some studies analysed the TF/TFPI expression balance mainly in the villous trophoblast [Estellés 1994, Aharon 2004], although a recent report focuses on the expression of whole sections of the placenta [Aharon 2005]. Placental TFPI is decreased in gestational vascular complications and can be restored by maternal enoxaparin treatment.

However, TF is produced and induced in monocytes, endothelial cells, fibroblasts, and macrophages, in addition to villous throphoblasts. We first investigated the gene expression in the placenta “in toto” (Fig 1), searching for possible different expression depending on the sampling site.

We were not able to show any difference between areas near cord insertion and each of the other sections. Then, another method of dissection (Fig 2) was used, but also in this case no different expression among the different samples analyzed was recorded.

PAI -2 is mainly expressed by human placenta tissue [Astedt, 1986], but also by macrophages, and a possible role in placental cell invasion in the uterus has been invoked [Zhou, 1997]. As for TF/TFPI, PAI-2 antigen has been previously studied in term healthy placentas and in those from preeclamptic women. We decided to study the whole section of normal placentas. It is clearly demonstrated that TF is expressed by trophoblasts in addition to placental macrophages or fibroblasts [Aharon 2004]. Our data show that TF is expressed in the whole section of placenta; in addition, immunohistochemical studies (data not shown) clearly demonstrate the localization of TF in at term syncytiotrophoblasts and in the adventitial layer of blood vessels.

We found a direct relationship between expression of TFPI or TFPI-2 and PAI-2. Furthermore, TFPI-2 expression is directly related also to TF.

These relationships could be responsible , at least in part, for local haemostasis in placentas from normal pregnancies. In agreement with Aharon and coll, who studied syncytiotrophoblasts culture , we can hypothesize also in at term placentas, the presence of a hemostatic balance critical for normal placental function and pregnancy outcome.

Our data show a relationship between VEGF and TF and TFPI-2 expression. Previous studies demonstrated that VEGF can stimulate the production of TF and, furthermore, that TF can stimulate expression of VEGF in human umbilical vein endothelial cells.

The presence of a relationship between TF and VEGF in at term human placentas was observed . In addition, we observed a relationship between VEGF and TFPI-2, suggesting

the presence of a counterbalance of the angiogenesis by TFPI-2, that might have an important role to maintain intervillous blood flow. These data are in agreement with those by Xu et al., who demonstrated in endothelial cells that VEGF induces both time- and dose-dependent increase in TFPI-2 mRNA.

In conclusion, our data show that sampling sites does not influence placental transcript expression. Studies aimed at the evaluation of the relationships here described in clinical conditions, as preeclampsia and IUGR responsible for an imbalance in the placental development, are needed.

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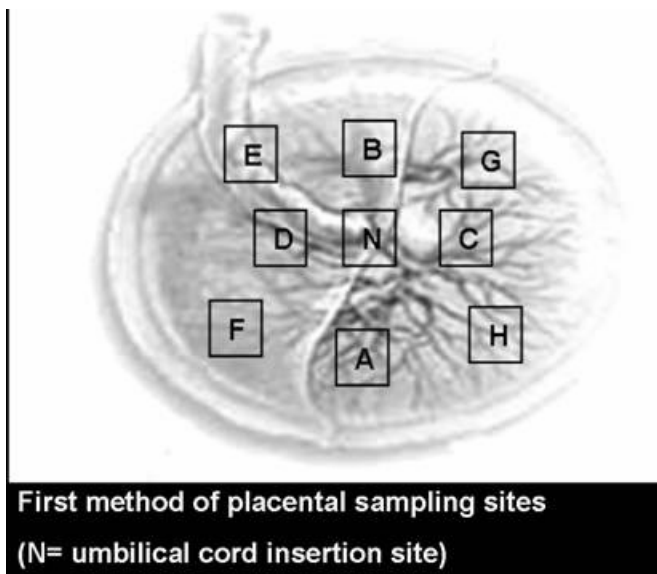


Figure 1

First method of placental sampling sites (N= umbilical cord insertion site)

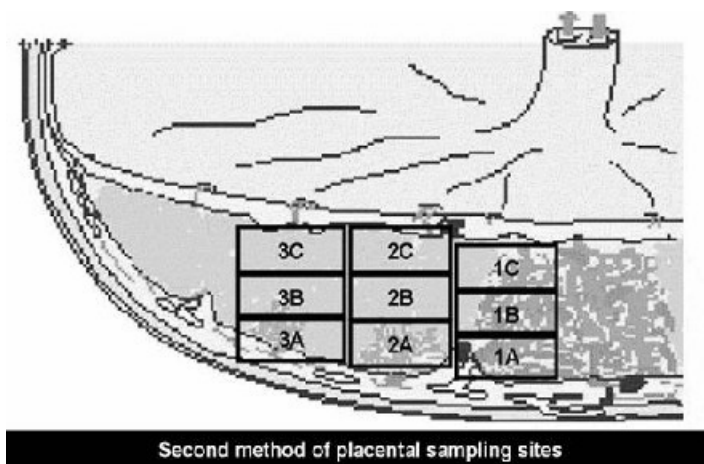


Figure 2

Second method of placental sampling sites. From each section 9 samples were obtained

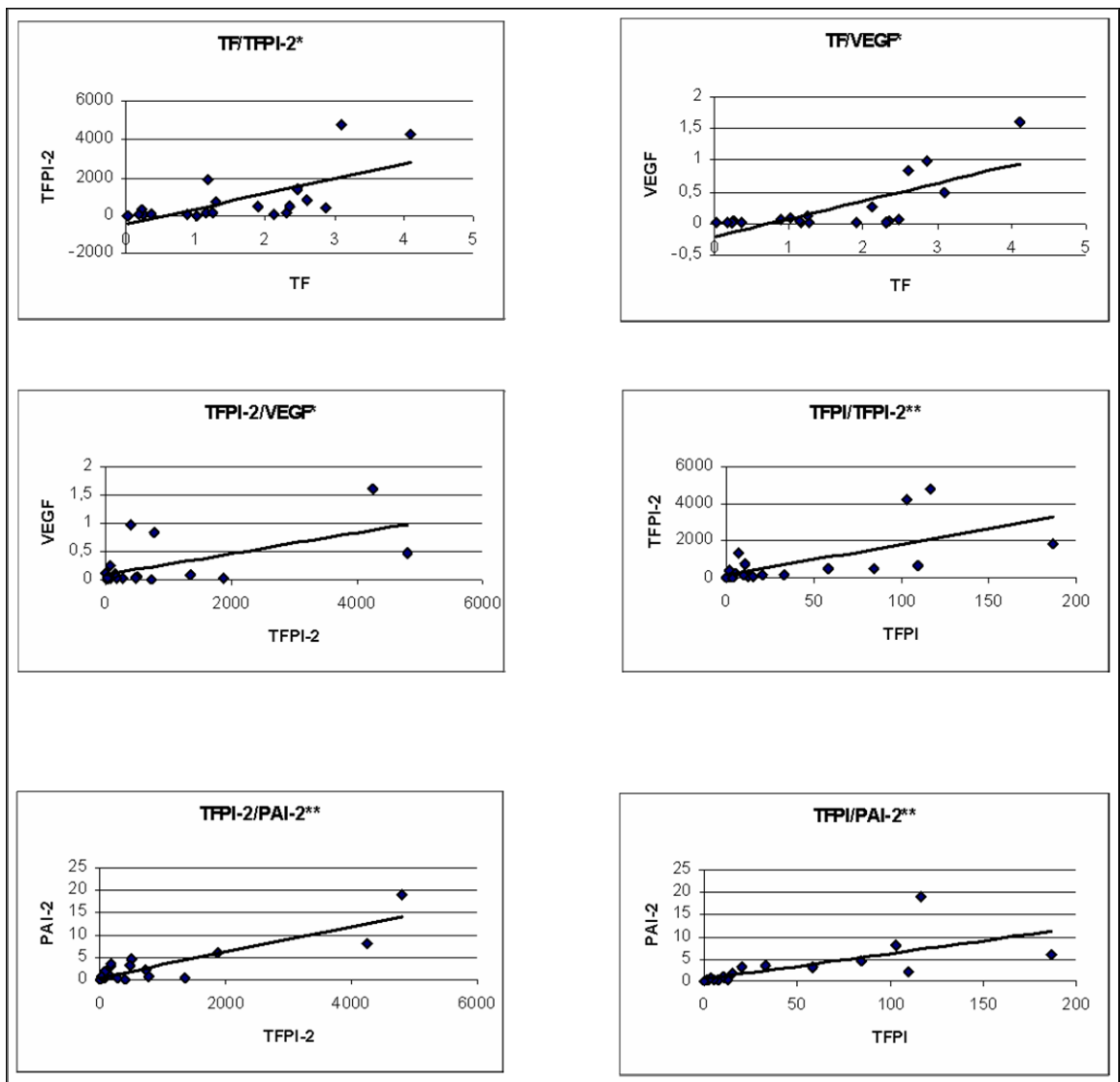


Figure 3

Statistically significant correlations

Spearman's correlation coefficients (n=21 placentas)

*p ≤ 0.05 level. ** p ≤ 0.01 level

Table 1 Gene expression in placenta , according to sampling sites as reported in Fig 1(ANOVA, p always > 0.05)

	TF [2 ^{-ΔCt}]	TFPI [2 ^{-ΔCt}]	TFPI-2 [2 ^{-ΔCt}]	PAI-2 [2 ^{-ΔCt}]	ANX V [2 ^{-ΔCt}]	VEGF [2 ^{-ΔCt}]	TM [2 ^{-ΔCt}]
N	2.35 ± 0.41	11.92 ± 15.31	362.48 ± 774.13	0.28 ± 0.20	3.52 ± 0.87	0.10 ± 0.07	0.10 ± 0.06
A	2.33 ± 1.71	6.80 ± 5.07	242.18 ± 181.20	0.20 ± 0.05	5.12 ± 2.18	0.66 ± 0.85	0.07 ± 0.04
B	2.83 ± 0.63	6.12 ± 4.11	82.71 ± 67.37	0.16 ± 0.08	4.09 ± 2.22	0.22 ± 0.24	0.09 ± 0.09
C	1.80 ± 1.48	18.91 ± 25.22	98.86 ± 84.09	0.29 ± 0.20	4.16 ± 1.74	0.10 ± 0.03	0.08 ± 0.04
D	2.53 ± 0.63	33.03 ± 5.26	1045.22 ± 1584.63	0.40 ± 0.69	6.06 ± 2.12	0.55 ± 0.62	0.28 ± 0.22
E	2.09 ± 1.18	19.73 ± 12.07	1293.06 ± 2241.37	0.63 ± 0.33	4.62 ± 2.44	0.53 ± 0.51	0.33 ± 0.34
F	3.55 ± 2.86	9.47 ± 6.78	234.25 ± 221.79	0.25 ± 0.12	5.71 ± 2.73	0.60 ± 0.45	0.06 ± 0.05
G	1.04 ± 0.41	6.83 ± 4.62	140.37 ± 129.37	0.75 ± 0.62	3.91 ± 0.59	0.52 ± 0.64	0.17 ± 0.25
H	6.50 ± 4.68	5.15 ± 3.35	444.33 ± 745.41	0.18 ± 0.35	5.65 ± 0.57	0.81 ± 0.37	0.12 ± 0.04

Table 2. Gene expression in placenta , according to sampling sites as reported in Fig 2 (ANOVA, p always >0.05)

	TF [2 ^{-ΔCt}]	TFPI [2 ^{-ΔCt}]	TFPI-2 [2 ^{-ΔCt}]	PAI-2 [2 ^{-ΔCt}]	ANX V [2 ^{-ΔCt}]	VEGF [2 ^{-ΔCt}]	TM [2 ^{-ΔCt}]
1C	0.49 ± 0.37	9.08 ± 6.91	188.23 ± 257.10	1.41 ± 1.28	10.65 ± 12.46	0.21 ± 0.28	0.29 ± 0.35
1B	0.42 ± 0.33	9.36 ± 6.56	246.32 ± 422.25	3.04 ± 3.13	9.90 ± 11.03	0.14 ± 0.16	0.24 ± 0.30
1A	0.58 ± 0.39	9.43 ± 6.71	385.29 ± 699.29	1.93 ± 1.42	13.10 ± 19.17	0.27 ± 0.34	0.24 ± 0.29
2C	0.49 ± 0.36	13.28 ± 8.14	140.71 ± 188.56	1.81 ± 1.46	13.17 ± 13.52	0.11 ± 0.11	0.29 ± 0.40
2B	0.62 ± 0.56	11.71 ± 8.80	251.66 ± 452.43	2.67 ± 2.49	15.75 ± 16.89	0.42 ± 0.76	0.77 ± 0.49
2A	0.78 ± 0.65	9.65 ± 7.60	252.40 ± 457.37	2.20 ± 2.30	9.55 ± 7.91	0.24 ± 0.32	0.17 ± 0.19
3C	0.52 ± 0.42	12.38 ± 8.41	97.04 ± 105.00	2.32 ± 2.04	16.23 ± 21.93	0.16 ± 0.21	0.30 ± 0.45
3B	0.58 ± 0.45	11.20 ± 8.42	157.41 ± 223.30	2.96 ± 3.22	22.90 ± 31.86	0.16 ± 0.23	0.31 ± 0.41
3A	0.54 ± 0.31	10.93 ± 7.21	59.02 ± 62.58	2.73 ± 2.57	16.44 ± 15.59	0.20 ± 0.35	0.34 ± 0.46

Submission in process

App. 3

EXPRESSION PROFILE OF DIFFERENT COAGULATION MARKERS IN HUMAN PLACENTAS FROM COMPLICATED PREGNANCIES

INTRODUCTION

Pre-eclampsia and IUGR are a serious complication of pregnancy that are potentially life threatening for both the mother and baby.

Risk factors for preeclampsia have been analyzed in a recent systematic review and include: a previous history of preeclampsia, primiparity, obesity, family history of preeclampsia, multiple pregnancies, and chronic medical conditions such as long-term hypertension or diabetes.

The hypothesized etiologic factors could be classified, at least, into 4 groups: genetics, immunologic, nutrition and infections, and interactions among them. The hypothesized pathophysiologic alterations are numerous, including failure of invasion of the trophoblast cells, oxidative stress, endothelial dysfunction, aberrations in calcitrophic hormones, release of growth factors, and antiangiogenic proteins among others. These changes may lead to proteinuria and hypertension and other phenotypes of the clinical syndrome of preeclampsia.

Intra uterine growth retardation is usually caused by uteroplacental vascular insufficiency and results in chronic fetal hypoxia, abnormal placental morphology, eccentric cord insertion. As such, local tissue hypoxia in the placental bed has been proposed as the mechanism limiting trophoblast invasion into the spiral arteries.

The placenta is a unique organ with dual blood circulation functioning throughout fetal development. The architecture and functions of the placenta, where maternal blood flows in the intervillous space, present haemostatic problems, mainly the risk of haemorrhage. Placental trophoblasts express and produce coagulation components, participating not only in haemostasis, but also in the placental vascular development and differentiation. The expression of tissue factor (TF), membrane phosphatidylserine and fibrin render the trophoblasts pro-coagulant, thus compromising the risk of bleeding while exposing the placenta to pro-thrombotic risks. Local inhibitory mechanisms TFPI-1 and TFPI-2, thrombomodulin, annexin V and the fibrinolytic system limit coagulation activation and fibrin deposition. Pregnancy complications have been associated with abnormalities in the functions of these inhibitors. Haemostatic processes in placental cells change throughout gestation and are affected by the changing requirements of the organs.

The aim of our study was to investigate the associations of the expression of some placental factors in women with successful pregnancy outcome and in women with onset of the preclampsia or other complications of the pregnancy.

MATERIAL AND METHODS

Placental samples of women with successful pregnancy outcome and women with preclampsia or other complications of the pregnancy; delivery during 35-42 weeks of gestation. Samples were frozen to -80°C .

The frozen samples of placentas were lyophilized and total RNA was extracted by means of Invitrogen Trizol Reagent, according to the manufacturer's instructions. RNA was treated with DNase (2 U of RNA), incubated in Gene Amp* PCR System 2400 Thermal

Cycle (Applied Biosystems) for 20 minutes at 37 °C, 3 minutes at 85 °C, 10 minutes at 4 °C. Electrophoresis on a 1% agarose gel was performed to confirm the presence and quality of RNA. RNA concentration and purity were determined by UV absorption at 260 nm and 280nm. Complementary DNA (cDNA) was prepared from the total RNA by means RT-PCR (Promega Reverse transcription system).

mRNA expression

Quantity mRNA expression of TF, TFPI, genes were evaluated by ABI 7700TM quantitative real time PCR system (Applied Biosystems, Warrington, UK) and compared to the human housekeeping glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) gene. Primer and probe sequences were either supplied as a ready mix (for human GAPDH and for human TF, human TFPI). Each sample contained: 3 µl of cDNA, Taqman Universal PCR Master Mix (12.5 µl), GAPDH primers and probe mix 20x (0.625 µl), target primers and probe mix 20x (1.25 µl) and RNase free water to a volume of 25 µl. Amplification was performed for 10 min at 95 °C, 45 cycles of 15 seconds at 95°C, 60 seconds at 60 °C. Gene expression levels were calculated using standard curves generated by serial dilutions of cDNA. A strong correlation between PCR efficiency of the internal control (GAPDH) and the target allowed the use of the ΔC_t -method to quantify comparable mRNA levels. Three independent analyses were performed with replicates.

The relative gene expression values were calculated using the comparative Ct method. the amount of target, normalized to GAPDH is given by $2^{-\Delta}$

RESULT

TF and TFPI expression were evaluated in 9 preeclamptic women and in 21 controls. Expression of TF and of the inhibitor (TFPI) were minor in the patients with pregnancy complicated by PE. The mean of TF in patients with PE is 0,88 (SD 1,04); in the uneventful pregnancies the mean is 1,7 (SD 1,23); the mean of TFPI in group of PE is 7,63 (SD 10,61); in control with uneventful pregnancy the mean is 39,89 (SD 51,35). TFPI values in pregnancy complicated by PE result fivefold inferior vs uneventful pregnancy.

DISCUSSION

The incidence of preclampsia (PE) is 5-10% of all pregnancies and this disease is a major cause of feto-maternal morbidity/mortality. The real cause of PE is unknown.

Preclampsia was defined as maternal blood pressure of more than 140/90 mm Hg with proteinuria (.300 mg/24 h or 12 on single urine sample) and edema. Several causes (genetic, nutritional, immunologic, infectious) and pathological mechanism were proposed (abnormal placentation, oxidative stress, endothelial damage). In any case all these causes and all these mechanism may interact and all may be responsible of the syndrome. Many case-control studies suggest a possible correlation between pro-thrombotic factors and poor outcome of pregnancy, while other authors don't show this association. The real mechanism responsible of the syndrome were not well known. PE, IUGR, placental abruption, fetal death were linked to a low flow in feto- maternal circulation because deficit of trophoblastic invasion and remodelling of spiral uterine arteries. In the last years several studies shown that the thrombotic factors acquired or

hereditary may cause a low blood flow leading thrombosis in feto-placental unit.

Evidences to support this hypothesis derived principle from association of study which revealed high prevalence of pro-thrombotic factors in coorti of pregnant with poor outcome of pregnancy.

Even if these studies were conducted with some cases, these dates were utilized for the women with poor outcome of the pregnancy; these women received treatment with heparin in successive pregnancy. Others studies didn't show high levels of thrombotic factors in population with poor outcome. For these authors the pro-thrombotic factors the utility of the heparin therapy will be appropriate only after the unequivocally benefit showed by randomize clinical trial.

In this study RNA was extracted and then cDNA was obtained by means RT-PCR and the expression of TF, TFPI, TFPI-2 was evaluated. The result must be correlated with successive studies which will evaluate expression of these factor in different areas of placenta syncitio and extracellular matrix because we now have dates given from all placental tissue. We observed a minor genetic expression of TF, but the potential thrombosis is secondary to the low levels of the TFPI.

The value of genetic expression of some placental thrombotic factors and the blood or urinary sample of these would be utilized to evaluate the start and or the severity of preeclampsia /IUGR. Naturally if we know the value of these factors we will utilize in high risk population for prevention of thrombotic accident.

We want in the future to evaluate genic expression of this and other prothrombotic factors in different histological placental sections such as syncytiotrophblast and extracellular matrix.

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App. 4

PREGNANCIES COMPLICATED FROM PRECLAMPSIA and IUGR: EXPRESSION PROFILE OF COAGULATION MARKERS IN PLACENTAS

Introduction

Preeclampsia is a multisystem disease of unknown etiology associated with increased perinatal/maternal morbidity and mortality. Although preeclampsia is commonly associated with intrauterine fetal growth restriction (FGR), small-for gestational-age infants (SGA) can also be observed without hypertensive disease, and the severity of FGR is correlated with the degree of placental infarction and impaired placental nutrient transport.

Physiologic changes in the coagulation and fibrinolytic systems are known to result in an increased susceptibility of pregnant women to thrombotic disorders and the formation of fibrin deposits, which can lead to a reduction in nutrient transport across the placenta (Estellés, 1994).

In a preliminary study, performed on a group of 28 placentas from uncomplicated pregnancies, we found a direct relationship between expression of TFPI or TFPI-2, two inhibitors of TF, and PAI-2. Furthermore, TFPI-2 expression was directly related also to TF. These relationships showed a correlation between coagulation and fibrinolytic systems and could be responsible, at least in part, for local haemostasis in placentas from normal pregnancies. In the same study was found a relationship between TF and TFPI-2 and VEGF, one of the principal regulators of angiogenesis (Wulff, 2003). So in placenta

from uncomplicated pregnancies there was a relationship between haemostasis and angiogenesis.

The expression of TF renders the trophoblasts pro-coagulant thus compromising the risk of bleeding while exposing the placenta to pro-thrombotic risks. Local inhibitory mechanisms, TFPI and TFPI-2, TM, Anx V, and the fibrinolytic system, limit coagulation activation and fibrin deposition. Pregnancy complications have been associated with abnormalities in the functions of these inhibitors (Lanir, 2003).

Human placenta is characterized by deep trophoblast invasion and complete remodeling of maternal vasculature by digestion of upper parts of myometrial spiral arterioles. VEGF, PlGF, Ang-1 and Ang-2 appear not only to regulate vascular development and remodeling during placentation but also act as growth factors for driving growth and differentiation processes such as trophoblast invasion and maturation failure of trophoblast invasion is associated to preeclampsia and IUGR. (Wulff, 2003).

The aim of this study is to know if the previously described relationships, found in uncomplicated pregnancy, fail to exist in placentas from preeclamptic or FGR pregnancy. We can hypothesize the presence of a hemostatic balance critical for normal placental function and pregnancy outcome.

In order to verify our hypothesis we first evaluated the expression of factors involved in haemostatic and angiogenic mechanisms in these complicated pregnancy and compared them with expression levels in uncomplicated one.

Materials and methods

Placental tissue acquisition

Placentas were obtained from four groups of pregnant women: a control group (n=21) and three groups whose pregnancies were complicated by normotensive IUGR (n=20), PE (n=10) or PE+IUGR (n=17). Control cases were not complicated by IUGR, PE or other conditions such as HELLP, diabetes mellitus and renal disease. PE was defined by maternal proteinuria >300mg/L in a 24 h collection together with blood pressure in a previously normotensive woman >140/90 mmHg on two or more occasions. IUGR was identified by newborn weight <3rd percentile of appropriate birth weight for gestational age (SMILA Neonatal standards for North-East Italy; Montecatini SEP 1996) (Table 1). Placental tissues were sectioned into samples of approximately 2cm x 2cm x whole thickness taken from the area closest to the cord insertion and stored at -80 °C.

From some placenta in complicated and uncomplicated groups (n=8 from PE, n=5 from C) immediately after delivery cytotrophoblasts were isolated.

Syncytiotrophoblasts cell culture

Cells were isolated as previously described (Kliman 1986) with some modifications. Villous tissue from the deciduas side was cut into small pieces. Tissue (~ 50g) was washed with 0.9% NaCl at room temperature and digested by three steps in warmed Hanks' solution (Cambrex..) containing 0.125% trypsin (Sigma...) and 0.2 mg/ml DNase I (Sigma ...). Supernatant with 10% FCS (Cambrex..) was layered on Histopaque 1077 (Sigma) and centrifugated (1200 rpm for 20minutes). Cells in the median layer were separated, washed and plated on culture plates (35mm) pre-coated with fibronectin

(VWR..). Cells were cultured in M199 (Cambrex) supplemented with 50 µg/ml gentamicin, 1% penicillin-streptomycin (cambrex), 20% (vol/vol) FCS and incubated in humidified 5% CO₂-95% air at 37°C. After the first 24 hours, apoptotic syncytiotrophoblasts were washed out and the remaining cytotrophoblasts differentiated and matured in culture into new generation of syncytiotrophoblast. After 14 days cells were characterized with cytokeratin 7 by.....

From cytokeratin 7 bind cells RNA was extract by means of Invitrogen Trizol, Reagent (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's instructions.

RNA purification, reverse transcription and quantitative PCR

The frozen samples of placentas were liophylized and total RNA was extracted by means of Invitrogen Trizol, Reagent (Invitrogen Corporation, Carlsbad, California) according to the manufacturer's instructions. RNA was treated with DNase (2 U /µg of RNA), incubated in Gene Amp PCR System 2400 Thermal Cycler (Applied Biosystems, Warrington, UK) for 20 minutes at 37 °C, 3 minutes at 85 °C, 10 minutes at 4 °C. Electrophoresis on a 1% agarose gel was performed to confirm the presence and quality of RNA. RNA concentration and purity were determined by UV absorption at 260 nm and 280 nm. Complementary DNA (cDNA) was prepared from the total RNA by means RT-PCR (Promega Reverse trascription system) (Promega, Madison, Wisconsin, U.S.A.). Quantity mRNA expression of TF, TFPI, TFPI-2, PAI-2, Anx V, TM, VEGF, PlGF, Ang-1, Ang-2 genes were evaluated by ABI 7700TM quantitative real time PCR system (Applied Biosystems, Warrington, UK) and compared to the human housekeeping glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) gene. Primer and probe

sequences were either supplied as a ready mix, TaqMan® Gene Expression Assay (VIC-MGB for human GAPDH and FAM-MGB for human TF, human TFPI, human TFPI-2, human PAI-2, human ANX V, human TM, human VEGF, human PIGF, human Ang-1, human Ang-2). Each sample contained: 3 µl (0.04 µg/µl) of cDNA, Taqman Universal PCR Master Mix (12.5 µl), GAPDH primers and probe mix 20x (0.625 µl), target primers and probe mix 20x (1.25 µl) and RNase free water to a volume of 25 µl. Amplification was performed for 10 min at 95 °C, 45 cycles of 15 seconds at 95°C, 60 seconds at 60 °C. Gene expression levels were calculated using standard curves generated by serial dilutions of cDNA. A strong correlation between PCR efficiency of the internal control (GAPDH) and the target allowed the use of the Δ Ct-method (14) to quantify comparable mRNA levels. Three independent analyses were performed with replicates.

Statistical Analysis

Statistical analysis was performed by means of SPSS. All the results are presented as mean \pm SD . ANOVA was used to compare two or more independent groups. Correlation between parameters was assessed using the Spearman rank test. Values of $p < 0.05$ were considered significant.

Results

Our data show that haemostatic factors TF, TFPI, Anx V and PAI-2 are respectively 2, 8, 1.5 and 5 fold less expressed in placentas from complicated pregnancy (n=47) than in control (n=21) ones ($p < 0.01$). On the other hand angiogenic markers (VEGF, PIGF, Ang-1; Ang-2) were not significantly different.

Dividing the complicated group in PE with or without FGR group (n=27) and FGR group (n=20), we found that in both groups TF, TFPI and PAI-2 were significantly ($p \leq 0.01$) lower expressed in respect of controls. In PE group TF, TFPI and PAI-2 expression were respectively 0.5, 6 and 4 fold lower than in control; in FGR group they were respectively 3, 2 and 6 fold lower than in control. Anx V was 2 fold less expressed in FGR than in control ($p=0.01$), but in PE the expression was not statically different, on the other hand TM was 2 fold higher than in control ($p < 0.01$), but not in FGR.

In PE group Ang-1 and Ang-2 were 2 fold higher expressed than in control ($p < 0.01$). In FGR group Ang-2 was 3 fold lower than in control.

Syncytiotrophoblasts data.

Syncytiotrophoblasts cells data show that TFPI-2 was 5-fold less expressed in complicated group in respect of control ($p < 0.05$). The other haemostatic factors were not significantly lower expressed. Among angiogenic factors only VEGF was significantly lower (10 fold) expressed in complicated group ($p < 0.05$).

Correlations between haemostatic factors

In all complicated groups there was a significant ($p < 0.05$) direct correlation, as in control group, between TF and its inhibitors: TFPI and TFPI-2. Furthermore, both the inhibitors of TF were directly correlated with PAI-2 ($p < 0.01$). But analyzing separately the three complicated groups we found that in PE and in FGR groups the correlations between TF and its inhibitors fail to exist. (A new correlation was found in PE group between TFPI and Anx V).

Correlations between angiogenic factors

About angiogenic mechanisms in placentas from uncomplicated pregnancies we observed a significant direct correlation between Ang-1 and Ang-2 ($p<0.05$), PlGF and Ang-2 ($p<0.05$).

In PE there was significant indirect correlation ($p<0.05$) between Ang-1 and PlGF, this correlation was not found in FGR group.

Correlations between haemostatic and angiogenic mechanisms.

In placentas from uncomplicated pregnancies we observed a significant direct correlation between VEGF, TF and TFPI-2 ($p<0.05$). On the other hand, PlGF was not correlated with these two but there was a significant direct correlation ($p<0.05$) with TFPI, PAI-2 and Anx V. Ang-1 and Ang-2 were significant correlated with TFPI ($p<0.05$). TF was significantly correlated with Ang-1 ($p<0.05$) but not with Ang-2. TFPI-2, PAI-2 and PlGF were significantly correlated with Ang-2 ($p<0.05$) but not with Ang-1.

Analyzing the correlations between angiogenic and haemostatic mechanisms in complicated pregnancies we found that VEGF was not correlated with anyone of the analyzed haemostatic markers. In the complicated groups separately we found in PE with or without FGR a significant direct correlation between PlGF and TFPI-2 ($p<0.05$), PAI-2 ($p=0.01$), Anx V ($p<0.01$). Also in FGR group there was a significant direct correlation between PlGF and PAI-2 ($p<0.05$). In all complicated groups (PE, PE+IUGR, IUGR) Ang-1 was significantly directly correlated with TF. In PE and PE+IUGR there were significant indirect correlation ($p<0.05$) between Ang-1 and PlGF and PAI-2, these correlation were not found in IUGR group. Ang-2 was correlated only with TF in IUGR group.

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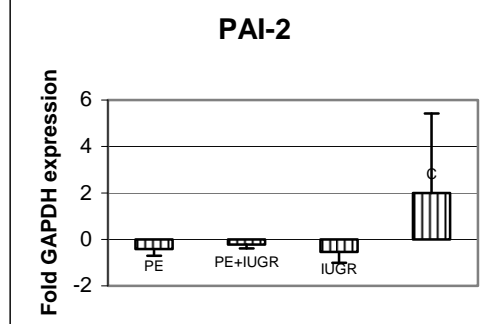
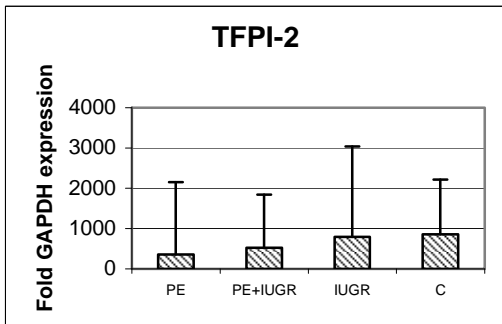
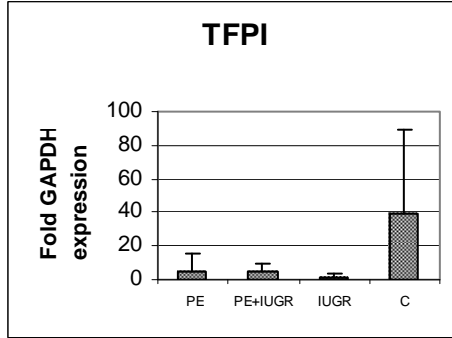
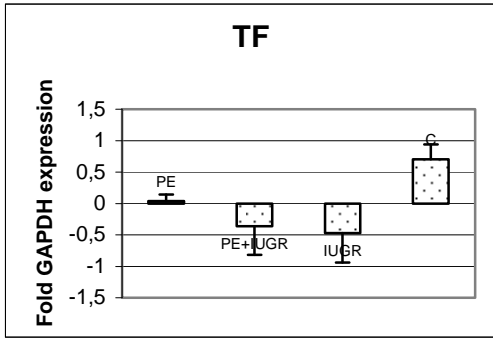
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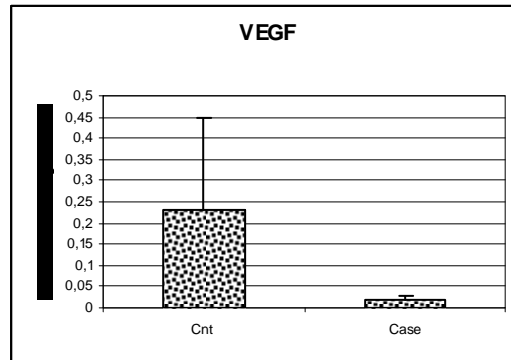
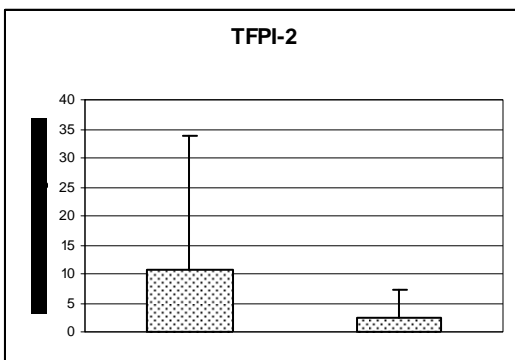
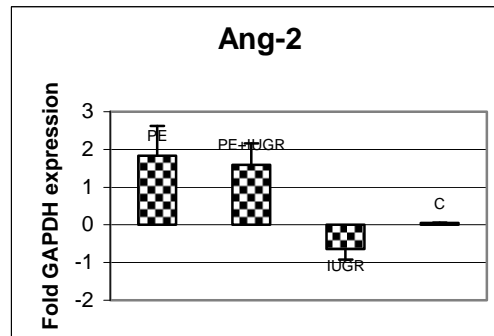
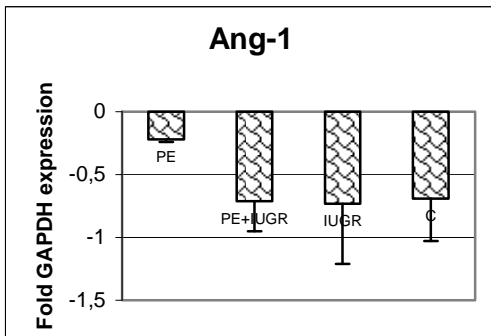
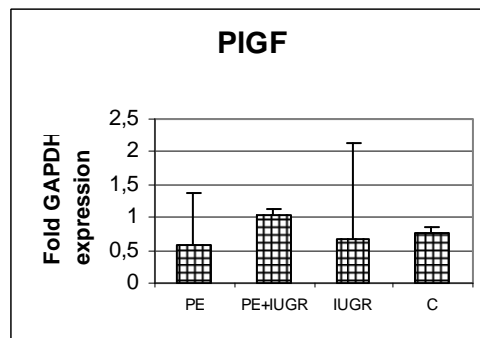
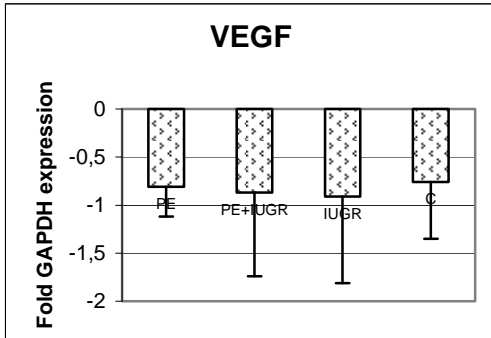
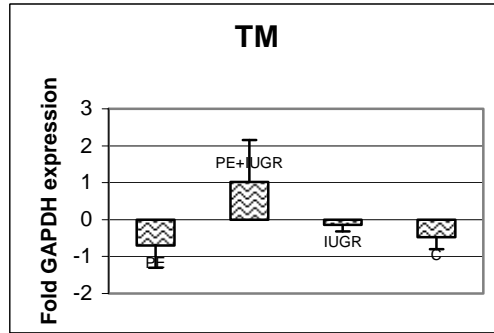
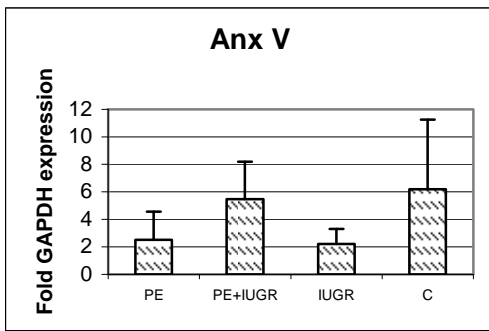
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Gene Symbol	Assay ID	Context Sequence	Chromosome
TF	Hs00175225_m1	CCTGGAGACAAACCTCGGACAGCCA	1
TFPI	Hs00196731_m1	TTGCAACAAGAAAAGCCAGATTTCT	2
TFPI2	Hs00197918_m1	ACCAAAGAAAATTCCATCATTTTGC	7
PAI 2	Hs00234032_m1	AGATGGCCAAGGTGCTTCAGTTTAA	18
ANXV	Hs00154054_m1	GCCATGGCACAGGTTCTCAGAGGCA	4
VEGF	Hs00173626_m1	ATGCCAAGTGGTCCCAGGCTGCACC	6
TM	Hs00264920_s1	GAGCAGCAGTGCGAAGTGAAGGCCG	20
ANGPT1	Hs00375823_m1	GAAAAAACAGTTTATTAGAACATA	8
ANGPT2	Hs01048043_m1	CCACATCAAACCTCAGCTAAGGACCC	8
PIGF	Hs01119262_m1	CGAATGCCGGCCTCTGCGGGAGAAG	14

	<i>Weeks' gestation</i>	<i>Birth wt (g)</i>	<i>Percentile</i>	<i>Placenta wt (g)</i>	<i>Maximal diastolic blood pressure (mm Hg)</i>	<i>Proteinuria mg/L in 24 h</i>
PE (n=10)	33±3,36	2117±893,25	25<p<50	600±100	95±6	1474±1083,99
PE+IUGR (n=17)	33±2,56	1251±449,87	3<p<10	314±149	98±10	9860±12901,37
IUGR (n=20)	33±4,84	1373±765,13	3	300±212	99±11	<300
C (n=21)	39±1,73	3319±445,08	50<p<90	506±70,71	76±7	<300





Syncytiotrophoblast gene expression

Next Submission on Placenta

PLACENTA, THROMBOPHYLIA, DOPPLER

Pre-eclampsia is a serious complication of pregnancy that is potentially life threatening for both the mother and baby. It encompasses a number of abnormalities that may be present in other clinical conditions. A placenta is essential for the development of pre-eclampsia and can be important in the pathogenesis of pre-eclampsia. Normal pregnancy is associated with remodelling of the maternal spiral arteries, which deliver blood to the placental villous space. Remodelling involves invasion by placental cytotrophoblasts that cause the maternal spiral arteries to lose their smooth muscle and become capacitance vessels; this process, known as placentation, is complete by 20 weeks of pregnancy. Poor placentation is associated with small-for-gestational-age fetuses and some cases of pre-eclampsia. It is thought that poor placentation can result in a hypoxic placenta that releases 'toxic substances' into the maternal circulation, contributing to the maternal syndrome. A number of candidate 'toxic substances' have been proposed, but none is universally raised in pre-eclampsia. Although the placenta is necessary for the development of pre-eclampsia, the extent to which placental abnormalities contribute to the condition varies. It is becoming apparent that maternal constitutional factors may also be important in this syndrome. Underlying hypertension, diabetes and obesity strongly predispose to pre-eclampsia. However, a continuum of risk may exist for blood pressure, bodyweight, glucose and lipids, which, in combination with each other and some degree of placental abnormalities, may lead to the development of pre-eclampsia.

A regular placental invasion and a normal placental morphology are indispensable for a normal pregnancy and a normal fetal development. In International literature there are many papers on placenta and its genetic, biochemical and hormonal aspects; many reports outline the relationship between placenta and pregnancy's diseases or its relationship with fetal malformation. Today we again don't know the modifications directly responsible of some disease (IUGR and PE) and of the fetal malformations; we will try to found the mechanism responsible of these diseases and its link with placenta valuing the correlation of anomalies Doppler ultrasonographic waves with the expression of the placental factors.

The physiological changes that occur during pregnancy create a hypercoagulable milieu. This hypercoagulable state is thought to be protective, especially at the time of labor, preventing excessive hemorrhage. The presence of hereditary or acquired causes of thrombophilia during pregnancy tilts the balance in favor of unwanted venous thromboembolism and adverse pregnancy outcomes due to vascular uteroplacental insufficiency. These adverse pregnancy outcomes include recurrent pregnancy losses, intrauterine fetal death, intrauterine growth retardation, preeclampsia and placental abruption. Much of the current data with regards to the association of the different thrombophilias and pregnancy-related complications are based on retrospectively designed studies. Recurrent pregnancy loss affects 1% to 3% of women of reproductive age, and a large proportion of these losses remain unexplained. Thrombophilic defects were found in 49% to 65% of women with pregnancy complications compared with 18% to 22% of women with normal pregnancies, suggesting a 3- to 8-fold increase in risk

Few studies have documented thrombotic lesions observed on the pathologic examination of the placenta in women with severe pregnancy complications. Moreover, a significantly higher rate of factor V Leiden and prothrombin G20210A gene mutations have been found in placentas with thrombotic events compared with normal placentas. In addition, clinical studies have been performed, using Doppler ultrasonography, to assess the uterine placental circulation in women with thrombophilia. Most of the Doppler studies of the umbilical and uterine arteries in pregnancies with thrombophilia were performed in women with antiphospholipid antibodies. Few Doppler studies also suggest improved uterine placental circulation when women with thrombophilia received thromboprophylaxis.

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We show the adverse outcome in women with thrombophilia and bilateral uterine artery notches considering the decreased uteroplacental blood flow during pregnancy in carriers of prothrombotic mutations.

App. 5

Outcome in women with thrombophilia and Bilateral uterine artery notches: our experience

Discrepancies about a possible relationship between prothrombotic mutations and a decreased uteroplacental blood flow are present in literature. We prospectively evaluated a cohort of pregnant women (n = 41) with bilateral uterine artery notches and found that women with inherited thrombophilia showed a sixfold higher risk to have an adverse outcome than women without.

Women with decreased uteroplacental blood flow during pregnancy have been found to be carriers of prothrombotic mutations, but there are some discrepancies on this issue (1,2). We prospectively evaluated a group of women with bilateral notch of uterine arteries at 23 weeks of gestation. The presence of inherited causes of thrombophilia was investigated. FV Leiden, FIIA20210 mutations were evaluated during pregnancy, and levels of natural inhibitors (protein C, protein S, antithrombin) were measured 3 months after the delivery. Forty-one women (mean age 29.1 ± 4.9 years) were enrolled and fetomaternal outcome was recorded. They were followed at the University “Federico II” of Naples and underwent the same obstetric surveillance during the current pregnancy. All women were tested for natural anticoagulants 3 months after the delivery.

Institutional Review Board approval was obtained. Gravidity and parity (mean \pm SD) were 1.5 ± 0.7 and 0.5 ± 0.7 , respectively. Overall, 21 (51.2%) women were nulliparous. Among the parous women, 3 (15%) reported a previous small-for-gestational age newborn, 3 (15%) an intrauterine fetal death, 6 (30%) early pregnancy losses, and 8 (40%) an uneventful pregnancy. None of the women suffered from chronic or preexisting disorders, such as diabetes or hypertension. Six (14.6%) of the 41 women showed inherited thrombophilia (2 heterozygous for FV Leiden, 3 heterozygous for FII A20210, and 1 protein S deficiency). Features of thrombophilic and nonthrombophilic women are shown in Table 1. Among the women with thrombophilia, 4 (66.6%) showed an adverse obstetric outcome (1 intrauterine fetal death, 2 small-for-gestational age newborns with a weight below the fifth percentile, 1 severe preeclampsia). Among the nonthrombophilic women ($n=35$), 9 (25.7%) showed an adverse outcome (6 small-for-gestational age newborns, 3 intrauterine fetal deaths), with an odds ratio of 5.8 (95% confidence interval: 0.9–37.4). A recent study evaluated the presence of an association between common prothrombotic factors and increased blood flow resistance in the fetomaternal circulation, in addition to the occurrence of obstetric complications (2) The investigators did not find an association between thrombophilia and blood flow in the fetomaternal circulation in nulliparous women. The design of our study is different from that of Salomon and colleagues (2), and we believe that comparison between data is difficult when different study designs are used. We did not study only nulliparous women, although 68.3% of women ($n=28$) were at their first pregnancy. Moreover, we decided to not evaluate the prevalence of the MTHFR 677TT homozygosity, as data about its role as a thrombophilic risk factor are not consistent.

TABLE 1
Features of thrombophilic and nonthrombophilic women

Feature	Thrombophilic women (n= 6)	Nonthrombophilic women (n= 35)
Age (mean \pm SD)	29.6 \pm 5.5	29.3 \pm 5.3
Parity	0.3 \pm 0.8	0.5 \pm 0.7
Gravidity	0.5 \pm 1.2	1.5 \pm 0.7
Smoked during pregnancy	0	7 a
a All smoked 1–10 cigarettes/day		

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Submission On Congress PRECLAMPSIA 2007

PLACENTA, THROMBOPHYLIA and HIGH RISK PREGNANCY

The link between thrombophilia and adverse outcome of the pregnancy was well known in literature; we present a case of a patient with Protein C deficiency and Budd Chiari syndrome.

App. 6

We report after the management of the thrombophilia in pregnancy considering our experience and the experience of international literature.

App. 7

Pregnancy in a woman with a history of Budd-Chiari syndrome treated by porto-systemic shunt, protein C deficiency and bicornuate uterus

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Case Report

Pregnancy in a woman with a history of Budd-Chiari syndrome treated by porto-systemic shunt, protein C deficiency and bicornuate uterus

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Budd-Chiari Syndrome (BCS) is a rare disorder caused by the obstruction of hepatic venous outflow, mainly due to the thrombosis of hepatic veins or of the terminal portion of the inferior vena cava (1). Although many cases are considered "idiopathic", underlying inherited (deficiency of natural coagulation inhibitors, factor V Leiden and prothrombin G20210A mutations) or acquired (myeloproliferative disorders, antiphospholipid syndrome, paroxysmal nocturnal haemoglobinuria, pregnancy, oral contraceptive use), conditions leading to thrombophilic states are often detectable and concur to the pathogenesis of BCS (1–7). Due to the rarity of the disease and to the low number of women conceiving after BCS, few data are available concerning counselling, management and outcome of pregnancy in this setting.

Case report

We report the case of a 34-year-old woman admitted to our institution at 10 weeks of her first pregnancy. Ten years earlier, she underwent a porto-systemic shunt after BCS, and was then prescribed life-long oral anticoagulant therapy, as protein C deficiency (42%, normal values 70–120%; STA[®] functional Protein C, Diagnostica Stago, Asnières-sur-Seine, France) was diagnosed. Screening for other congenital (plasma antithrombin, protein S and homocysteine levels; activated protein C resistance; genotyping for factor V Leiden and prothrombin G20210A mutations) or acquired (lupus anticoagulant and anticardiolipin antibodies; objective and laboratory data suggesting haematological, immunological or chronic inflammatory disorders) thrombophilic conditions was negative. Type I protein C deficiency was then confirmed by repeated measurements, also including evaluation of antigen levels (46%, normal values 68–125%; ELISA with rabbit polyclonal antibodies, Dako A/S, Glostrup, Denmark) and ruling out possible causes of acquired deficiency (other indexes of liver biosynthesis and vitamin K-dependent proteins were within normal ranges).

Warfarin had been withdrawn after the biochemical diagnosis of pregnancy (6th week) and substituted by nadroparin 5,700 UI twice daily. Ultrasonography showed a bicornuate uterus, with a gestational sac and vital embryo in the right uterine horn and a large intramural leiomyoma in the other uterine horn.

Ultrasound follow-up during pregnancy did not show abnormalities of the liver and of the hepatic vein flow, presence of fluid in abdomen or foetal malformations and growth restriction. Biochemical testing of liver enzymes, bilirubin, platelet count and coagulation parameters (PT, aPTT; activated protein C resistance; plasma fibrinogen, antithrombin, D-dimer) were in the normal range throughout the pregnancy. Ultrasound and laboratory follow-up was performed every 3–4 weeks until the 20th week, then every two weeks. Pregnancy was uneventful until 29 weeks of gestation, when the patient was admitted because of severe swelling of the legs, ascites and a premature rupture of membranes (PROM), which prompted labour induction and the need for urgent caesarean section because of podalic presentation. At the opening of the abdominal wall, about five liters of milky ascites were found. The male newborn (weight about 900 g; 1 and 5 minutes Apgar scores: 1 and 5, respectively; venous and arterial umbilical pH: 7.0–7.1) died after two days because of respiratory distress, although the lack of necropsy did not enable to rule out other possible reasons for his death. As expected due to the neonate's prematurity, coagulation tests showed abnormally prolonged PT and aPTT and low plasma levels of coagulation factors and inhibitors, including protein C.

Discussion

BCS is a rare and serious thrombotic disease with significant morbidity and mortality. The underlying acquired and/or inherited thrombophilic conditions, often associated with this syndrome, contribute to the severity of prognosis, with the risk of thrombotic recurrences and co-morbidity in patients with haematological or immune disorders and cancer (1–7).

Early diagnosis and intervention to reduce portal hypertension, together with prolonged anticoagulation and the possibility of liver transplantation [a 68% overall survival at ten years has been recently reported (8)], have improved clinical outcomes and life expectancy of patients with BCS. Therefore, conceptional and pregnancy issues in fertile women should be carefully addressed.

The association of BCS with pregnancy is well recognised, several cases having been reported since the early 1970's (9–10).

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On the other hand, very few data are available concerning pregnancy in women with a history of BCS (11–12).

It is known that pregnancy can result in fatal complications for the mother and/or for the infant in women with chronic liver disease (13). Fertility is often decreased in advanced liver disease, and this may provide a degree of protection for such patients who would be at increased risk; however, pregnancy may occur even in the presence of advanced liver disease, and it is necessary to anticipate and plan for possible complications of the specific liver disease. Counselling prior to pregnancy is the best policy, with consideration of transplantation prior to childbearing or of sterilization if it is more appropriate, based on the patient's general and liver function evaluation (13). In this respect, the presence of severe portal hypertension and high risk of variceal bleeding (further increased during pregnancy) may play a relevant role. In women with BCS, risks related to the possible coexistence of thrombophilia and to anticoagulation also have to be taken into account, as in the patient here reported. This woman was carrier of protein C deficiency, a severe inherited thrombophilic condition (14). Increased risk of venous thromboembolism is reported during pregnancy and puerperium in patients with inherited deficiency of coagulation inhibitors, and such risk is likely to be also greater in women with previous thromboembolic events than in asymptomatic carriers (15–16). Antithrombotic prophylaxis with low-molecular-weight heparins (LMWH) has been shown to be a safe and effective approach for women carrying thrombophilic abnormalities and/or with a history of venous thromboembolism in pregnancy (17–18). In this respect, preconceptional advice may include the withdrawal of coumarins prior to childbearing, also in order to prevent the risk of embryopathy (19).

Our patient started her pregnancy without such counselling, and the presence of uterine abnormalities (bicornuate uterus with a large intramural leiomyoma in the left horn) was unknown. Bicornuate uterus is the most frequent congenital uterine abnormality (20), whose prevalence is not negligible in the gen-

eral female population (0.4–4%). In such a condition, the inadequate vascularity to the developing embryo and placenta, the reduced uterine intraluminal volume or cervical incompetence predispose to pregnancy complications [early miscarriage, preterm labour and breech presentations (20)].

To our knowledge, this is the first pregnancy described in a woman with a history of BCS treated by porto-systemic shunt and with a bicornuate uterus. The association of the two diseases is only coincidental and contributed to the unsuccessful outcome of pregnancy. The physiologic enlargement of the uterine horn during pregnancy caused a compression of the shunt, leading to the onset of severe ascites and, presumably, to the PROM with labour induction. However, the relative contribution of the anatomic uterine abnormalities and of the ascites as factors triggering PROM cannot be established. The sudden and severe presentation of ascites was rather unexpected in this patient, who underwent a careful laboratory and ultrasound follow-up since the 20th week, and hampered the induction of enhancement of foetal lung maturity planned at 30–32th week.

Despite the adverse outcome for the foetus, this report supports the concept that pregnancy in women with previous BCS and porto-systemic shunt is possible, and that prophylaxis with LMWH is safe and effective also in pregnant women with severe thrombophilia, although the optimal regimen for prophylaxis and its monitoring (LMWH would require anti-factor Xa levels, often not available, as in this patient) are still subjects of debate (21). Due to the low number of women conceiving after BCS, recommendations and guidelines regarding future conception and management during pregnancy in this setting have not yet been defined. Our experience highlights the need for a careful preconceptional counselling in these women, including advice for liver and vascular disease, antithrombotic prophylaxis, especially when thrombophilic abnormalities coexist, and the search for other maternal disease potentially affecting pregnancy outcome.

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Management of thrombophilia in pregnancy

During pregnancy the risk of thrombosis is high. Thrombophilias are broadly defined as conditions predisposing to thrombosis with increment of development of gestational vascular complications such as first- and second trimester miscarriages, intrauterine growth restriction (IUGR), intrauterine fetal death, placental abruption, preclampsia. Many studies show a direct association between thrombophilia and pregnancy complications; for this reason the management of women with a profile of thrombophilia must be more accurate. The complications of pregnancy after thrombosis are the major cause of perinatal and maternal morbidity and mortality worldwide with high social and economic impact. Preconceptional counselling, familiar history and preventive treatments, including low-molecular weight heparins, aspirine, folate are considering in pregnancy and in puerperium. It will be necessary with accurate screening to value the presence of factor of thrombophilia and to choose with the patient the therapy considering collateral effects. Even if there are not prospective trials which quantified the risk of obstetric complications in women with thrombophilia, preliminar datas suggest that the prophylaxis of coagulation may ameliorate outcome in women with thrombophilia. Multidisciplinar approach is another fundamental question. We analyse aspects of the question considerino the modern literature.

Discussion

Thrombophilia is a common factor risk with a prevalence of 15-25% in white population; the term thrombophilias encompasses both inherited and acquired conditions, continuous or transient with high risk of thrombosis; thrombophilias have particular relevance in pregnancy due to the coexisting physiological alterations in the coagulation system that occur during pregnancy and which result in an increased tendency toward thrombosis. (Tab. I) These disorders increase the risk of thrombosis during pregnancy with risk of vascular complications. Risk of thrombosis is major of 5-6 fold during pregnancy, with same frequency in the three trimester of pregnancy and 3-10 fold major in post-partum. Thrombophilias is present in 50% of women with thrombosis correlated to the pregnancy. Sometimes the discovery of thrombophilic state occur in pregnancy. Placental pathological findings in women with thrombophilia are hallmarked by thrombosis and fibrin deposition with reduction of placental flow. Obstetric major complications as fetal loss, IUGR, preclampsia, abruptio occur in 1-5% of women. (1-2)

Hemostasis is regulated by a delicate equilibrium between factor procoagulant and factor anticoagulant. Alterations of any type may alter this equilibrium predisposing to thrombosis. Also in placenta there is this equilibrium; the slow placental flow and the high coagulation state during pregnancy with stasis that occur in area of poor perfusion may predispose to thrombosis and obstetrical complications. Even if the knowledges on therapy during pregnancy are poor, datas in literature suggest that some groups (women with personal or familiar history of thromboembolic disease) will be screened for thrombophilia. Test are necessary (Tab. II) also in women with history of fetal loss in first trimester, severe preclampsia, intrauterine growth retardation IUGR, abruptio

placenta, intra uterine fetal death because always more knowledge show association with thrombophilic factors. (Tab. III)

Tab I. Prevalence of inherited and acquired thrombophilic disorders (1)				
Type	Defect	% General Population	% Patients 1° TVP	Risk TVP (OR)
Inherited				
-AT III	Reduction of inactivation of thrombin	0,07	1	10-20
- protein C deficiency	Reduction of inactivation FVa , FVIIIa	0,3	3	6-8
- protein S deficiency	Reduction of inactivation FVa , FVIIIa	0,2	3	2-6
- Factor V Leiden (Etero)	Reduction of inactivation FVa Protein C attiv	5-8	20	4-8
- Factor V Leiden (Omo)	Reduction of inactivation FVa Proteina C attiv	0,06	1,5	80
- Prothrombin gene mutation	High levels of thrombin and prothrombin	3	6	2-4
- hyperhomocysteinaemia	Endothelial disease	5	10	2-3
- Methylenetetrahydrofolate reductasi mutation	Reduction of metilation of omocysteinaemia	10-20	11-12	0,7-2
Acquired				
- Antiphospholipid syndrome	Altered pathway Protein C, Annessina V loss, endothelial procoagulant effects	2	10-15	9
- Activate Proteina C resistance (without FV Leiden)	Reduction of anticoagulant activity	8-11	24	2-4

Tab. II Evaluation of laboratoristic risk factors of thrombosis (3)
Test I level Evaluacion of protein C activate resi stance or analysis of DNA of Factor V Analysis DNA for mutation of prothrombin gene Omocysteinemia levels Lupus anticoagulant Antibody anticardiolina Emocrome Test II Level Functional evaluation of AT III Functional evaluation of protein C Functional evaluation of protein S Test III level Levels of fibrinogeno Levels of Factor VIII Time of thrombin Reptilase time Test IV level Analyss of DNA for mutation C677T of MTHFR Levels of factors V, VII, IX, X, XI Levels of trombomodulina Levels of tissutal activator of plasminogeno Levels of di heparin cofactor II Major patients will be know with I and II level test; the III level test in the cases of familiar thrombosis when other test aren't useful for diagnosis. The IV level test are utilized for research.

Placental vascular complications associated with thrombophilia Tab. III (12)				
	Miscarriage	IUFD	Preclampsia	Placental abruption
Deficit Antitrombina III	++	++	+	
Deficit Proteina C	++	++	+	+
Deficit Proteina S	++	++	+	+
Disfibrinogenemia	++	+		
protein C Resistance	++	++	++	+
Factor V Leiden	++	++	++	++
MTHFR677TT	+	+	+	+
Iperomocysteinemia	+	+	++	++
Factor II G20210A	+	+	+	++
Antiphospholipid syndrome	++	++	++	++
Combined defects	++	++	++	++
+ = Possible association ++ = Association sure				

In many women with inherited thrombophilia the pregnancy will finish without complications even if the risk of obstetrical disease will be elevated. The real motive because only in some patient will occur the obstetrical disease is unknown, even if we think and the literature too, that many other factors systemic or local and placental play a fundamental role.

The presence of more thrombophilic factors increase the thrombotic risk of 14 fold versus a single factor which increase the risk of 4-fold. (1)

Actually 30-50% of vascular diseases of pregnancy are linked to the thrombophilia.

We must discover if new factors thrombophilic congenital or acquired may play a role

(thrombomodulin, gene of endothelial receptor of protein C, deficit of factor XII, deficit

of protein Z, cofactor of the X activated, high levels of procoagulant microparticell) (1).

For this motive the decision is to not include all patients in the screening for thrombophilia,, because the real benefits of this choose aren't supported from this politic.

(4). The presence of inherited thrombophilia, didn't change the dose of therapy because the deficiencie of AT, protein C and protein S, the factor V Leiden and the mutation of prothrombin aren't resistant to the anticoagulant therapy.

The presence of this thrombophilic factors may change, increasing the time of administration, especially after one evnt of thrombosis. The optimal time of therapy depend from a balance between risk of thrombosis without therapy and risk of haemorrhage during the administration of anticoagulant; the therapy must be accurate for each patient. (5)

Women with history of thrombotic accident, generally are in therapy with dicumarolo inhibitor of vitamin K, with effect teratogen well known; during the preconceptional must be outlined the importance of stop the therapy when there is a positive test of pregnancy; at the same time must be outlined the importance of start a therapy with low molecular weight heparin (LMWH) or fractionated heparin.

Thromboembolism venous and pulmonary embolia are rare but serious complication during the pregnancy and are the principle cause of morbidity and mortality in Europe and in USA. The number of deaths for million of pregnancy is changed in the last 20 years. In any case the incidence of thromboembolic venous disease is 0,5-0,7 / 1000 delivery while the incidence of pulmonary embolia is 1/2500- 1/10000 (in the case of caesarean delivery the risk show an increase of 3-fold) 6).

The thrombotic events are the same in pregnancy and in post-partum with a distribution in the three trimester. The major incidence of death will be occur in the first two weeks after delivery and the 40% 2-6 weeks after the delivery, so after dimission. The risk factors for TEV are numerous and would evaluate in all pregnancies. (Tab. IV).

Risk factors for thromboembolism in pregnancy and in puerperium (<i>Tab. IV</i>) RCOG 2004 (30)	
<p><i>Preesistent</i></p> <p>Precedent history of thromboembolism</p> <p>Trhombophilia</p> <p>-inherited: Deficit AT III, Deficit Protein C/Protein S, Fattore V Leiden, variant prothrombin</p> <p>- acquired: Antiphospholipid syndrome, lupus anticoagulant</p> <p>Ab anti cardiolipina</p> <p>Age > 35 anni (BMI > 30 Kg/ m2)</p> <p>Parity > 4</p> <p>varicous disease</p> <p>Paraplegia</p> <p>sickle cell disease</p> <p>Infections</p> <p>Mieloproliferative diseases</p>	<p><i>New or transient</i></p> <p>Surgery in pregnancy or puerperium</p> <p>Operative delivery</p> <p>Immobility > 4 days</p> <p>Disidratation state</p> <p>Haemorrhage</p> <p>Iperemesi</p> <p>Ovarian hyperstimulation syndrome</p> <p>Pre-eclampsia</p> <p>Long labour</p>

For the thrombophilia and Antiphospholipid syndrome there is a grade of risk that change considerino the defects; the anomalies with high risk are: deficiencie of AT III (30%), omozigous for Factor V Leiden, the positivity for antibody antiphospholipid and the presence of combined antibody. For the prevention of the thromboembolic disease is fundamental the evaluation of therapy with anticoagulation and the possible diagnostic deeper.

In the cases of thromboembolic events (Familiar < 40 yearsi) before the conception will be useful the screening for inherited or acquired thrombophilia, to limit the periods

of immobility and the state of disorientation. In the presence of signs suggestive of antiphospholipid syndrome (thrombosis arterial/venous, miscarriage, thrombocytopenia) other tests as the research of Lupus anticoagulant and antibody anti cardiolipin are useful. The women with history of venous thromboembolism received ENF (50-100 u/kg in bolus followed from 1250-1500U/h in continuous infusion (aPTT ratio= 1,5-2) for 5-10 days followed from ENF every 12 h s.c. with dose accurate (aPTT ratio 1.5-2.5 a 6 h from each administration) to term and for 6 weeks in post-partum. Today a good alternative is of heparin LMWH 80-100 U/Kg every 12h in first phase and after 50-80 U/kg every 12h successively. For the ENF the dose will be reduced to 5000-7500 UI sc every 12h when the labour start or before the caesarean delivery. For the low molecular weight heparin it is necessary to stop the administration to the start of labour or 12 hours before caesarean delivery. Therapy must be stop 24h before induction of delivery; it is considered sure to stop the therapy in immediate pre-partum and postpartum.

Therapy may after restarted in EV (2000UI in bolus followed from 1200 UI/h in continuous infusion) or in s.c. and it may continue until restarted of oral therapy.

Women with positive history for thromboembolic event have a risk of recidive of 4-15%; in these women the optimal plasmatic levels of heparin are 0,08 - 0,15 UI/ml (median dose 16400 UI/die) (7).

Potential advantage of LMWH heparin on ENF are the major antithrombotic action, a minor risk of haemorrhage a single injection for emilife more long, minor dose of the heparin, minor thrombocytopenia, minor risk of osteoporosis. (10) A collaborative study of the 1998 showed the safety and the success of heparin LMWH 90% of women with a history of recurrent pregnancy loss and in 100% of women with preclampsia in the

precedent pregnancy. (11) The administration of Enoxaparin 20mg/die showed a good success until 80% of live birth in patient with a history of recurrent miscarriage (12). The administration of Enoxaparin 40mg/die showed a success of 75% of live birth at term in a group of patients with history of thrombophilia versus only 20% in pregnancies of the same group in women without therapy

This success was observed also in other studies not randomized. (Tab. V) (13-14)

Observational studies for prevention of poor prognosis of pregnancy in women with thrombophilic diseases (Tab. V) (13-14, 27)					
	N° women	Defect	history	Therapy	Alve born
- Brenner 2000	50	Congenital/acquired	RPL	Enoxaparin /ASA	46/61
- Grandone 2002	25	FV o FII	RPL; IUGR; PE	UFH/LMWH/ASA	29/31
- Kupfermanc 2001	33	Not specified	Obstetrical complication	Enoxaparin 40mg + ASA	30/33
- Riyazi 1998	26 vs 19	Congenital or acquired	Obstetrical complication	Enoxaparin 40 mg + ASA	Peso >
- Younis 2000	7	Factor V Leiden	RPL recurrent	Enoxaparin 40 mg + ASA	5/7
- Carp, 2003	37 vs 48	Congenital	RPL	Enoxaparin 40 mg	70% vs 40%

ENF was the drug of choice for the prophylaxis and the therapy of venous thromboembolism in pregnancy; this drug does not cross the placenta with no potential teratogenesis or haemorrhage for the fetus. (15)

Maternal complications reported during the therapy have an incidence of 3% (osteoporosis, thrombocytopenia, allergic disease and haemorrhage (16).

Low molecular weight heparin are safe for the fetus because they do not cross the placenta and have an efficiency and safety comparable with the heparin ENF.

Data on the more accurate dose are not available, and we choose the dose on the basis of the weight. In high risk pregnancy for thrombotic event a possible solution was to utilize a dose of Dalteparin 5000 UI every 24h in women with weight < 100 Kg and 5000UI every 12h in women with weight > 100 Kg. The rate of complications (vertebral fracture for osteoporosis) is the same of the heparin ENF (2,2%). (17)

A meta-analysis of Sanson on the safety of low molecular weight heparin LMWH in pregnancy report an incidence of poor outcome in the patients with associated diseases of 13% and only 3% in patient without diseases. (18) If we consider the haemorrhagic complications of the patients that utilize heparin LMWH versus the patient that do not utilize this therapy, it was observed a major risk of haemorrhage during delivery and major incidence of anemia (19)

Protamina sulfate was the drug contrasting heparin when haemorrhage starts (the dose is 1 mg every 100 UI; for the nadroparin it is necessary half dose.

Oral anticoagulant may cross placenta causing teratogenesis (skeletal dysplasia, nasal hypoplasia). Incidence of 5-10% between 6a 12a week of gestation. For many authors the risk of miscarriage is high in contrast of the patient that didn't receive heparin. (20)

Other malformations reported are malformation of SNC and of the eyes. The risk of teratogenesis is probable for the high dose of drug; some data show that a dose <5 mg may be safe. These data must be confirmed. (21) As this drug does not cross in the milk it may start again in puerperium. Aspirin is a safe drug when used in pregnancy in low dose (50-150 mg). There is no study showing a significant difference versus placebo in incidence of preclampsia, preterm delivery or intrauterine death. (22-23)

We must again establish if aspirin has a role as the heparin. The prevalence of thrombophilia, placental tissue deposition of fibrin, risk of recurrence are a good reason for the start of trials on the prophylaxis with anticoagulant to ameliorate the outcome of women with thrombophilic state and fetal loss recurrent. To support this hypothesis the better outcome of women with Antiphospholipid syndrome receiving antithrombotic therapy; the rate of live birth is major in women receiving heparin plus aspirin (80%) in comparison of women receiving only aspirin (40%) (24-25) Women with thrombophilic state and obstetrical diseases showed better Doppler flow of the uterine arteries in the next pregnancies after therapy with enoxaparin and aspirin. (26) We report some guidelines.

Tab.VI Guidelines for prevention of thromboembolism and use of heparin in pregnancy (7, 8, 30)

SISSET (2002)	ACCP (2004)	RCOG (2004)
a) Low-moderate risk: control clinic/instrumental, elastocompression, fast mobility	a) TVP + transient risk: look and prophylaxis (LMWH) postpartum	a) Precedent TVP no thrombophilia LMWH only post partum. Antepartum if TVP recurrent, risk factors no transienti, familiarità for TVP
b) High risk no thrombophilia: LMWH 2000-5000 UI/die sc last weeks and puerperium + a)	b) TVP + thrombophilia or familiarity: prophylaxis antepartum and postpartum (LMWH)	b) Precedent TVP + thrombophilia LMWH antepartum and post-partum
c) High risk + thrombophilia: LMWH 2000-5000 UI/die sc during pregnancy considerino the weight + a) + b)	c) Numerous TVP prophylaxisi antepartum + major period of postpartum (LMWH)	c) thrombophilia without TVP prophylaxis antepartum and/or postpartum considerino risk factors
	d) Antiphospholipid syndrome LMWH + ASA	d) women with 4 or more risk factors eventually antepartum e 3-5 days postpartum LMWH

In puerperium we must continue with LMWH or oral anticoagulant. The prophylaxis for women at risk is not well established for the insufficient evidences and for the data of expert opinion and no trials.

Even if the few trials compare the prophylaxis with placebo or 2 methods of prophylaxis, it is hard to establish if the therapy is efficient because low number of patients. (9)

Optimal dose of low molecular weight heparin must again be established; new trials are necessary.

Therapy indicated from RCOG (Tab. VII)

Tab. VII Prenatal prophylaxis and therapy with LMWH. RCOG 2004 (30)			
Prophylaxis	Enoxaparin (100U/mg)	Dalteparin	Tinzaparin
Normal weight (50-90Kg)	40 mg/die	5000 U/die	4500 U/die
Weight < 50 Kg	20 mg/die	2500 U/die	3500 U/die
Weight > 90 KG	40mg/12h	5000 U/12h	4500 U/12h
Major dose	40mg/12h	5000 U/12h	4500 U/12h
Therapeutic dose	1mg/kg 12h	90 U/Kg 12h	90U/kg 12h

Randomized multicentric controller trials will compare two dosage of enoxaparin in women with recurrent fetal loss. Therapy with 40 mg and dose with 80mg are equally efficacious with live birth of 81% and 77% compared with only 28% in precedent pregnancies.

Incidence of preclampsia and abruption placenta are more low. (27-28) In any case aren't accurate trials showing the real efficacy of heparin on the out come of pregnancy in women with thrombophilia. (29)

A trial placebo-controls evaluating the real benefits of heparin is ethically wrong and impossible.

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Congress PRECLAMPSIA 2005

APOPTOSIS and PRECLAMPSIA

Pregnancy complications such as preeclampsia, intrauterine growth retardation (IUGR) preterm labor affect a considerable number of pregnancies and account for significant perinatal morbidity and mortality. Although the pathophysiology has not been clearly defined, the common phenomenon observed between these diseases is abnormal development and function of the placenta (1–2). Normal placental development is dependent upon the differentiation and invasion of the trophoblast, the main cellular component of the placenta that originates from the trophoectoderm of the blastocyst early in pregnancy. During this process of differentiation and invasion, trophoblast cells rapidly divide to form the interface between mother and embryo, while other trophoblast subpopulations invade the decidua (pregnant endometrium) to remodel the arterial blood vessels in the uterine wall, known as the spiral arteries, to accommodate the expansion of extraembryonic tissue and to increase blood flow to the placenta and developing fetus. As a developing organ, the placenta undergoes constant. Tissue remodeling, which is characterized by the functional loss of trophoblast cells by apoptosis.

Apoptosis, or programmed cell death, is an active process by which superfluous or dysfunctional cells are eliminated to maintain normal tissue function. Depending on the stimuli, apoptosis may be initiated by one of two known pathways: intrinsically by the mitochondrial pathway and extrinsically by either the death receptor-mediated pathway or in response to exogenous stimuli such as cytokines (Fig.1). The central executioners of apoptosis are the caspases, which are a family of cysteine proteases that cleave numerous vital cellular proteins to affect the apoptotic cascade. Several endogenous inhibitors,

including fllice-like inhibitory proteins(FLIPs), inhibitors of apoptosis (IAPs), and antiapoptoticBcl-2 family members, inhibit caspase activation, there by preventing further propagation of the death signal. Whereas apoptosis is thought to be important for normal placental development, it may also be involved in the pathological conditions associated with this organ. Apoptotic cell shave been identified in both the maternal and fetal compartments of the placenta during normal pregnancy, and the presence of these cells may be related to the stage of placental development, including the attachment and invasion of the trophoblast (3), spiral artery transformation, trophoblast differentiation and turnover, and parturition (4). In complicated pregnancies such as preeclampsia or IUGR, a greater incidence of trophoblast apoptosis has been observed(5-9), suggesting that alterations in the regulation of trophoblast apoptosis may contribute to the pathophysiology of these diseases.

In placentas from complicated pregnancies, shallow trophoblast invasion and inefficient spiral artery transformation have been observed (10-11), which may be partly due to the distribution and activation state of infiltrating macrophages

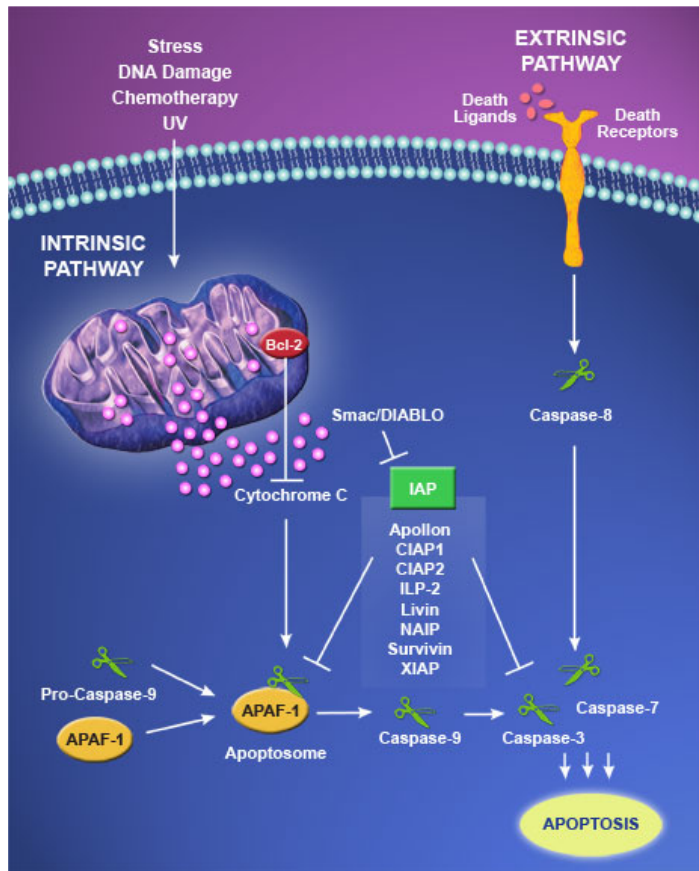


Fig.1 Two known pathways of apoptosis: intrinsically by the mitochondrial pathway and extrinsically by either the death receptor-mediated pathway or in response to exogenous stimuli.

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We evaluate morphogenetic alterations of placenta and apoptosis of trophoblast into placenta of pregnancies with preeclampsia/intrauterine growth restriction and controls.

App. 8

Morphogenetic alterations and apoptosis in placenta from pregnancies complicated by preeclampsia /intrauterine growth restriction: role of HGF/c-met/STAT3 and bcl-2.

Objective: Impaired trophoblast invasion is associated with early-onset Preeclampsia and intrauterine growth restriction. We evaluate morphogenetic alterations of placenta and apoptosis of trophoblast into placenta of pregnancies with preeclampsia/intrauterine growth restriction and controls.

Study design: Placental sections were obtained from women with early-onset preeclampsia/intrauterine growth restriction (n 11) and in controls (n18). Trophoblast's apoptosis into placenta was quantified evaluating bcl-2 apoptosis marker. Placental's morphogenetic alterations were evaluated with HGF/c-met/STAT3 cascade.

Results: Trophoblast apoptosis was not increased in preeclampsia/intrauterine growth restriction significantly ($P>0,05$); HGF/c-met/STAT3 cascade was completely altered in severe IUGR patients with placentas showing absence of peripheral ramifications ($P<0,05$).

Conclusion: This review focuses on the role of trophoblast apoptosis into placenta and show the role of HGF/c-met/STAT3 cascade in the onset of pregnancies with preeclampsia/intrauterine growth restriction.

Introduction

Preeclampsia is a potentially fatal complication of human pregnancy characterized by hypertension, proteinuria and edema. This disease complicates 5 to 10% of pregnancies and is a leading cause of maternal and fetal mortality and morbidity. (1) Intrauterine growth restriction defined as birth weight \leq 5th percentile or \leq 10th percentile for gestational age is a frequent cause of perinatal morbidity (2) and it may be a complication of hypertensive disorders such as preeclampsia . Although the cause of these diseases are unknown, inadequate invasion and remodelling of maternal uterine arteries by extravillous trophoblasts (EVTs) in the first trimester are a common feature. (3) Apoptosis, or programmed cell death, is an active process by which superfluous or dysfunctional cells are eliminated to maintain normal tissue function; this process is important for normal placental development, but it may also be involved in the pathophysiology of pregnancy-related diseases. Normal placental development is dependent upon the differentiation and invasion of the trophoblast, the main cellular component of the placenta. Trophoblast apoptosis increases in normal placentas as gestation proceeds but a greater incidence of trophoblast apoptosis has been observed in pregnancies complicated by preeclampsia or intrauterine growth retardation (IUGR).(4) The central executioners of apoptosis are the caspases, which cleave numerous vital

cellular proteins to affect the apoptotic cascade. By inhibiting caspase activation, several endogenous inhibitors, including the antiapoptotic Bcl-2 family members, can prevent further propagation of the death signal.

Hepatocyte Growth Factor (HGF) and its receptor, *c-met*, are essential components of intercellular signaling pathways for the control of growth and differentiation. This factor functions as mitogen, and morphogen for a variety of cultured cells and increases the invasiveness of epithelial cells. The human placenta is one of the original sources from which HGF was purified to homogeneity and the factor is now generally considered to be critical for placental growth and organogenesis. (5)

The etiology of preeclampsia is poorly understood; however, one characteristic of this disease is a lack of trophoblast invasion into the maternal decidual arteries; this decrease in arterial trophoblast invasion is thought to result in placental hypoxemia with subsequent production of placental factors that lead to the development of preeclampsia (6). Although the mechanism for decreased trophoblast invasion in preeclampsia is unknown, abnormal trophoblast integrin expression, insulin-like growth factors, and hypoxia have all been implicated in this process (7). In addition, a number of other cytokines, including transforming growth factor- β , epidermal growth factor, and interleukin-1, have been shown to regulate trophoblast invasion *in vitro*. (8) Another cytokine that may affect trophoblast invasion is hepatocyte growth factor (HGF) (9)

We hypothesized a concomitant role of an anomalous apoptosis process and the alteration of the HGF/*c-met*/STAT3 cascade with an anomalous placental morphology in the development of the preeclampsia and IUGR. A different grade of apoptosis was observed

in the patient with pregnancies complicated by preeclampsia and intrauterine growth retardation (IUGR) .

Material and methods

Placental tissues of gestation (n°29) were obtained by elective cesarean or spontaneous vaginal delivery from uncomplicated subjects (n° 18) and from patients with preeclampsia (n°3) and or with pregnancy complicated by intra uterine growth retardation(n°8). Uncomplicated subjects were normotensive throughout pregnancy and had no proteinuria or other signs of preeclampsia. Although these subjects included women undergoing spontaneous preterm labor, the cases with clinical chorioamnionitis (fever, maternal or fetal tachycardia, uterine tenderness, foul odor, and leukocytosis) were excluded. The gestational weeks of preeclamptic pregnancies ranged from 26-39 weeks. The gestational ages of all cases were determined by ultrasonographic examination in the first trimester. The mean birth weight in preeclamptic pregnancies was 1411.8 ± 811 g. Preeclampsia was established for systolic and diastolic blood pressure above 140 mm Hg and 90 mm Hg, on at least two occasions 6 or more hours apart, together with generalized edema or proteinuria. Proteinuria was defined as 300 mg or more.

Discussion

The human placenta is composed of mononuclear cells, cytotrophoblasts, which coalesce to become a multinucleated syncytium. Maintenance of trophoblast structure and differentiated function is essential for the provision of adequate gas, nutrient, and waste

exchange between the fetus and the mother. Placental trophoblasts may be subject to diverse insults during normal human pregnancy. Clinical conditions such as preeclampsia, anemia, smoking, and living in a high altitude can lead to placental underperfusion and villous hypoxia, characterized by diminished syncytial differentiation, syncytial knots, and prominent cytotrophoblasts. During the process of differentiation and invasion, trophoblast cells rapidly divide to form the interface between mother and embryo, while other trophoblast subpopulations invade the decidua (pregnant endometrium) to remodel the arterial blood vessels in the uterine wall, known as the spiral arteries, to accommodate the expansion of extraembryonic tissue and to increase blood flow to the placenta and developing fetus.

As a developing organ, the placenta undergoes constant tissue remodelling, which is characterized by the functional loss of trophoblast cells by apoptosis. After proliferation and differentiation into specific cell subtypes, aging trophoblast cells are selectively removed and replaced by a younger population of trophoblasts without affecting neighboring cells (10)

A greater incidence of villous as well as extravillous trophoblast apoptosis has been detected in placentas from pregnancies complicated by preeclampsia or IUGR, suggesting that the appropriate regulation of trophoblast apoptosis is important for normal pregnancy. (11-14). In response to different stimuli, apoptosis may be initiated extrinsically by the death receptor pathway or intrinsically by the mitochondrial pathway. The central executioners of apoptosis are the caspases, which cleave numerous vital cellular proteins to affect the apoptotic cascade. By inhibiting caspase activation, several endogenous inhibitors, including fllice-like inhibitory proteins (FLIPs), inhibitors of

apoptosis (IAPs), and antiapoptotic Bcl-2 family members, can prevent further propagation of the death signal. Macrophages present at the maternal-fetal interface may also contribute to trophoblast survival by removing apoptotic cells and producing cytokines and growth factors, which influence the progression of the apoptotic cascade.

(4)

Although some studies observed no differences in the expression of Bcl-2 in placental villi from complicated and normal pregnancies (15), a subsequent study reported that Bcl-2 expression was less abundant in syncytiotrophoblasts from severe preeclamptic and IUGR placentas than in the normal controls (16). Other authors demonstrated that the apoptotic extravillous cytotrophoblasts detected in preeclamptic samples were negative for Bcl-2 expression, suggesting that a decrease in Bcl-2 expression may induce apoptosis in extravillous trophoblast cells. (14, 17)

The group of patients with preclampsia showed a reduction of expression of the cascade HGF/c-met/Stat 3 with positive bcl-2 anti apoptosis markers. Placental morphology show a maturative stop in the process of throphoblast invasion. In the patients with IUGR, we observed villi with excessive ramification process and a soft reduction of the expression of the cascade. The bcl-2 markers was negative as expression of high grade of apoptosis.

We were not able to find any report in a MEDLINE search from 1966 using the key words apoptosis, HGF, preeclampsia.

Enhanced trophoblast apoptosis and anomalies of placental morphology has been observed in the placentas of women with preeclampsia and/or intra-uterine growth retardation.

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PLACENTA and FETAL MALFORMATION

Many fetal malformations as the neural tube defects (NTD) are linked to morphogenetic alterations due to a defective development of the embryonal phases. Hepatocyte growth factor (HGF)/c-met system plays a role in morphogenesis of nervous system, lung, and kidney. Hepatocyte growth factor, also called scatter factor (SF), is a multifunctional cytokine that promotes growth, differentiation, invasion, angiogenesis, and morphogenesis. HGF/c-met morphogenetic effects are mediated by signal transducers and activators of transcription (STAT)3 and both HGF and c-met genes are regulated from p53. A reduced mRNA expression of HGF/c-met/STAT3 pathway was found in the malformed nervous systems and placentas. On the contrary, detectable expression levels of p53, HGF, c-met, and STAT3 were observed in non-malformed fetus and in placenta of uncomplicated pregnancies. Many fetal malformation and pregnancy complicated with PE may be the linked for the presence of the same alteration of placental morphogenesis, with a reduction in all components of the p53/HGF/c-met/STAT3 cascade. (1) The placenta is a rich source of HGF/SF, and its absence in mice has been shown to lead to impaired placental growth and embryonic death (2)

A possible relationship between hepatocyte growth factor (HGF) expression and the pathogenesis of preeclampsia was evaluate in some studies; Furugori et al. measured the concentration of immunoreactive HGF and the expression of HGF messenger ribonucleic acid (mRNA) in human placentas obtained from two groups: uncomplicated and preeclamptic pregnancies at various gestational weeks. In addition, the localization of HGF mRNA and *c-met* protein was analyzed using *in situ* hybridization and

immunohistochemical staining, respectively. The expression of HGF mRNA and the concentration of immunoreactive HGF were highest in second trimester and were significantly decreased in preeclamptic placentas compared with the uncomplicated cases in third trimester. These results provide evidence of an abnormality of HGF expression in the preeclamptic placentas. Such placentas exhibit the abnormally shallow trophoblast invasion of the uterus, and reduced expression of HGF could well account for

this morphometric change. (3) Hepatocyte growth factor (HGF) is a cytokine that is produced in the placental villous core and acts in a paracrine manner on trophoblasts that express the HGF receptor Met. Because HGF stimulates the invasion of many epithelial cell types, villous core HGF could regulate placental trophoblast invasion. As preeclampsia is characterized by inadequate trophoblast invasion, Kauma et al investigated the hypothesis that decreased placental HGF production is a mechanism for inadequate trophoblast invasion in this disease. Placental villous explant HGF production over 24 h was 25% lower in patients with preeclampsia than in normal. These studies suggest that HGF has an important role in placental trophoblast invasion. In addition, decreased placental production of HGF in preeclampsia provides a potential mechanism for the lack of trophoblast invasion that is seen in this pregnancy disorder. (4)

Prenatally diagnosed malformation or anomalies of karyotype as trisomy 16 mosaicism are associated with the increased risk of poor pregnancy outcome including intrauterine growth restriction, intrauterine death and fetal malformation. While maternal preeclampsia has also been reported in some cases, this has not been systematically evaluated. To better define the risk of preeclampsia and the clinical course of preeclampsia in these pregnancies and to identify associated clinical variables, a study

reviewed 25 cases of prenatally diagnosed trisomy 16 mosaicism for which molecular studies were undertaken and sufficient obstetrical data were present to include/exclude the diagnosis of preeclampsia. Six of 25 (24%) mosaic trisomy 16 cases exhibited preeclampsia as compared to 3 of 44 (7%) matched controls. There were no differences between those mosaic trisomy 16 cases presenting with preeclampsia and those that did not, in terms of the presence/absence of UPD, IUGR, malformation, or trisomy on amniocentesis. The levels of trisomy tended to be high in placentas associated with preeclampsia; however very high levels of placental trisomy were also often seen in the absence of preeclampsia. As it is impossible to predict which subset of cases is at highest risk, all women receiving a prenatal diagnosis of trisomy 16 mosaicism should be closely monitored for signs of preeclampsia. (5)

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We have evaluated several placentas obtained immediately after delivery from normal (n°= 18), preeclamptic pregnancies (n°=11) (29–40 weeks gestation) and placentas of pregnancies complicated with fetal malformation. Immediately after obtaining the placenta, placental villi were dissected free from the decidua basalis, minced into small pieces, and rinsed free of blood. The placental villi (350 mg wet weight) were cultured in duplicate in 5 mL DMEM with 10 mmol l-glutamine, 100 mIU/mL penicillin, and 100 mg/mL streptomycin, pH 7.4 (Sigma Chemical Co., St. Louis, MO) for 24 h, and the medium was stored at -20 C until assayed for HGF. HGF in placental villous explant-conditioned medium was quantified by a sandwich ELISA for human HGF. We now are analyzing the dates from the placenta. Some of the cases of fetal malformation, for the particularity of the clinical features and the precocity of prenatal ultrasound diagnosis were reported.

“SPLIT NOTOCHORD SYNDROME VARIANT. PRENATAL FINDINGS AND NEONATAL MANAGEMENT”App. 9

“EARLY PRENATAL DIAGNOSIS OF CONCORDANT POSTERIOR URETHRAL VALVES IN MALE MONOCHORIONIC TWINS” App. 10

”MASSIVE FETAL HEMORRHAGE AND FETOMATERNAL ALLOIMMUNE
THROMBOCYTOPENIA FROM HUMAN PLATELET ANTIGEN 5B
INCOMPATIBILITY: AN UNUSUAL ASSOCIATION”..... App. 11

“DIAGNOSIS OF FEMORAL HYPOPLASIA-UNUSUAL FACIES SYNDROME IN
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PRENATAL DIAGNOSIS OF PLACENTAL CHORIOANGIOMA: OUR
EXPERIENCE.....App.13

PRENATAL DIAGNOSIS OF ARTHROGRYPOSIS.....App.14

CASE REPORT

Split notochord syndrome variant: prenatal findings and neonatal management

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Split notochord syndrome is an extremely rare form of spinal dysraphism characterized by a complete cleft of the spine and a persistent communication between endoderm and ectoderm. A variant of split notochord syndrome was diagnosed in a 25-week-old fetus showing a prolapsed congenital colostomy and a spinal cystic lesion. The final diagnosis included protruding colon segment, imperforate anus with a rectourethral fistula and lipomyelomeningocele. The ultrasound features of the condition and the post-natal management are discussed. The neonate was successfully treated with a posterior sagittal anorectoplasty, while the lipomyelomeningocele was resected at a later stage. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: split notochord syndrome; prenatal diagnosis; congenital colostomy; dorsal enteric fistula; lipomyelomeningocele

INTRODUCTION

Split notochord syndrome (SNS) represents an extremely rare and pleomorphic form of spinal dysraphism characterized by a wide spinal defect and a persistent communication between endoderm and ectoderm (Almog *et al.*, 2001; Gilbert-Barness and Luise, 1997). In its basic form, it consists of a neural tube defect with an endoectodermal fistula opening in the dorsal area, but several variants differing for the type and the site of the associated anomalies, which involve the gastrointestinal tract, the central nervous system (CNS) and, less often, the urogenital tract have been described by pediatric surgeons and neonatologist (Faris and Crowe, 1975; Flower, 1998). Owing to its pleomorphism, this condition has rarely been identified prenatally, with only two cases reported in the last 20 years (Almog *et al.*, 2001). We present here the prenatal findings leading to the putative diagnosis of SNS and the post-natal surgical management of this condition.

CASE REPORT

A 29-year-old obese woman, gravida 2 para 0, was referred to our centre at 25 weeks of gestation with a suspicion of a neural tube defect involving the distal part of the sacrum. The family history was unremarkable, with no consanguinity reported. The trans-abdominal ultrasound examination showed a small round hypoechoic apparently cystic mass involving the lower

part of the sacrum and an oblongated perineal structure closely resembling an intestinal loop, possibly originating from the lateral perineal region. None of the cerebellar and ventricular abnormalities usually associated with an open spinal defect were present. Since the fetus was in breech presentation and the patient significantly overweight, trans-vaginal ultrasound was performed. A much clearer view of the spinal mass, the intestinal fluid-filled structure and their relationship was achieved, the intestinal structure originating from the lateral aspect of the gluteal region (Figure 1A). The patient was informed about the likely presence of a combined defect of the caudal embryonal area involving both the spine and the lower intestinal tract, consistent with the split notochord spectrum of anomalies. Karyotyping was offered, but was declined by the patient. Two subsequent scans did not reveal any change in the volume of the masses or any other abnormality possibly overlooked at the first scan. At term, a male neonate weighing 3400 g was delivered by caesarean section.

Post-natal physical examination showed an intestinal loop originating in the lateral perineal area, with the serosa on its external surface (Figure 1B,C and Figure 2), a dorsal enteric fistula opening at the base of the loop, an imperforate anus and a skin-covered lump in the sacral area (Figure 1B and Figure 2). The left thigh was mildly hypoplastic and the right testicle was undescended. An X ray of the abdomen and spine showed the rectum ending blindly 3 cm from the perineal surface and the splitting of the sacrum and the coccyx with a scimitar-shaped sacral defect. A decompressive colostomy and a partial resection of the protruding colon segment were performed as initial treatment. Histology confirmed that the protruding structure was of intestinal origin and that the external layer was the serosa. The post-operative course was uneventful. Magnetic resonance imaging (MRI) and computed tomography (CT) of the

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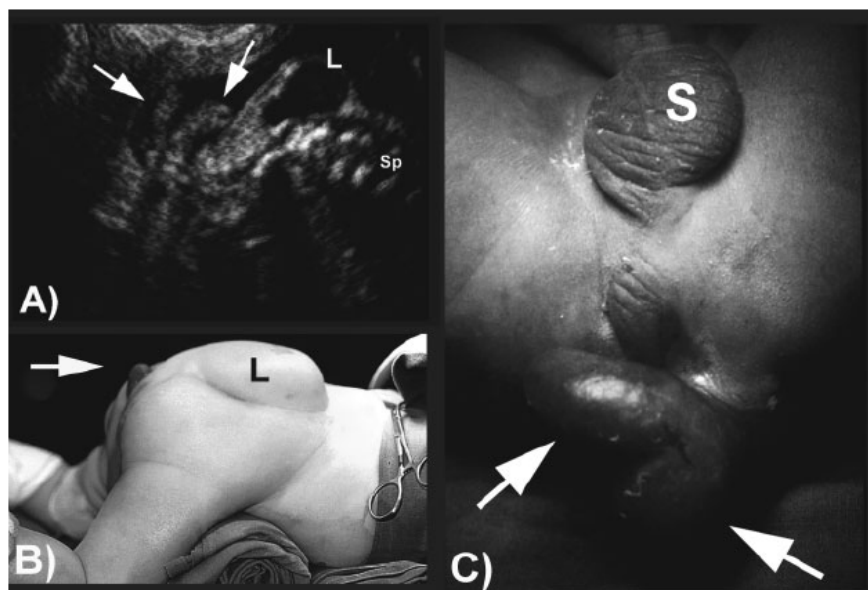


Figure 1—(A) At trans-vaginal ultrasound, the relationships between the protruding intestinal segment (arrows) and the sacrococcygeal lipomyelomeningocele (L) are demonstrated (Sp, spine). (B) For comparison, a lateral view of the lower trunk after the excision of the protruding intestinal segment and creation of the colostomy (arrow) is shown. Note the lumbar mass represented by the lipomyelomeningocele (L). (C) A frontal view of the neonate at birth showing the relationships between the intestinal segment (arrows) still in place and the scrotum (S)

brain and spine confirmed the normality of the former and the presence of a complete cleft below L₄ with a tethered cord associated with a lipomyelomeningocele. After counselling with neurosurgeons, it was decided to give priority to the repair the gastrointestinal malformation, and to untether the conus at a later stage. At the age of two months, the baby underwent posterior sagittal anorectoplasty via a combined dorsal and anterior approach. The double route approach was needed for the difficulties in locating the exact branching point of the protruding intestinal segment from the colon. During the intervention, a rectourethral fistula, demonstrated by the pre-operative distal colostogram, was also identified and closed. The protruding loop was then removed and the anoplasty completed in the standard way. The post-operative course was uneventful. The colostomy was closed at six months of age and the baby was discharged with a well-functioning anus. The colon was of normal length and size, whereas the ectopic sphincter showed a reduced para-sagittal muscular component, especially on the left side. The intestinal function was relatively good, with moderate constipation. As for the bladder function, the neurogenic bladder was managed by intermittent catheterization. At three years of age, the baby underwent the neurosurgical excision of the lipomyelomeningocele, in the hope that hopefully an improvement of bladder and intestinal function would ensue, but the situation remained basically unchanged after the operation.

DISCUSSION

Spinal dysraphisms consist of a wide spectrum of congenital anomalies resulting from defective embryogenesis of the spinal cord and vertebrae (Gilbert-Barnes and Luise, 1997). The pathogenesis of the condition is unknown. One hypothesis refers to the persistence or partial obliteration of an accessory neuroenteric canal that connects the yolk sac and the amniotic cavity in the third post-conceptual week (Rosset, 1995). However, the most accepted theory suggests that an anomalous splitting or duplication of the notochord would allow the endoderm or primitive gut to stick in and adhere to the dorsal ectoderm (Saunders, 1943). As for the lipomyelomeningocele, it is considered to be the result of the premature dysjunction of neuroectoderm and ectoderm (Sigh and Sigh, 1982).

The malformations featuring spinal dysraphisms can occur as isolated lesions or in combination with a wide range of anomalies of the spinal canal, the CNS and/or of other systems, most often the gastrointestinal tract. There are two varieties of spinal dysraphism, open and closed. In the former type (*spina bifida cystica*), the membrane and/or the cord may be exposed; in the latter type (occult spinal dysraphism), the spinal defect is covered with skin and can be associated or not with a skin-covered backmass (Byrd *et al.*, 1991). The SNS, together with the caudal regression syndrome, belongs to the group of complex occult spinal dysraphism (Tortoli-Donati *et al.*, 2000). Therefore, SNS represents an exceedingly rare



Figure 2—The dorsal view of the neonate shows the relationship between the protruding intestinal segment (arrowhead), the lipomyelomeningocele (L) and the scrotum (S)

malformation characterized by a persistent connection between the endoderm and ectoderm, resulting, in its most severe form, in a midline communication between the intestinal cavity and dorsal skin. In the most severe form, the so-called dorsal enteric fistula, the newborn has a bowel ostium on the back. Of note, SNS most commonly occurs in the lower cervical or upper thoracic spine, much less frequently in the lumbosacral region (Meller *et al.*, 1989), as in the index case.

The difficulties encountered in the effort to correctly classify the SNS variant of the index case are easily understood if a MEDLINE search is attempted. We performed a search in the English language literature (1966–2004) for the terms 'split notochord syndrome' and 'dorsal enteric fistula' and retrieved only nine affected children. However, the lesions are so different from each other with such a wide range of associated anomalies that each one might be considered a unique variant (Table 1).

With respect to prenatal diagnosis of SNS, there are only two such reports, and both describe SNS with thoracic openings (Almog *et al.*, 2001). To the best of our knowledge, this represents the first case of prenatal recognition of an SNS located in the lower spinal area. In our case, at ultrasound the occult spinal dysraphism (OSD) appeared as a sacro-coccygeal skin-covered cystic mass, thought to represent a simple spinal defect,

but post-natally recognized as a lipomyelomeningocele, while the perineal mass showed all the features of an intestinal loop, hence the putative diagnosis of a complex malformation involving tissues of ectodermal and endodermal origin.

The differential diagnosis of lipomyelomeningoceles includes the cystic variant of sacrococcygeal teratomas and *spina bifida*. However, the latter is associated in the majority of cases with secondary modifications of the central nervous system, and, in addition, the bony vertebral defect is almost always detectable. As for sacrococcygeal teratomas, they are generally much larger and are not associated with other perineal or gluteal anomalies. Isolated lipomyelomeningoceles can be detected prenatally (Seeds and Powers, 1988), but not in the context of spinal dysraphisms. Since SNS is often associated with tethered cord, neurological deterioration may ensue, even in asymptomatic children, if prophylactic surgery is not performed. Although the anomaly is grossly evident at birth, the prenatal recognition of the condition allows *in utero* transport to tertiary referral centres. Whenever unusually appearing structures of intestinal origin are detected in association with cystic lesions of the lower spine, SNS and, in general, open spinal dysraphisms are the most likely diagnoses.

A last comment about the prenatal diagnostic issue regards the possible advantages of three-dimensional ultrasound. Since this anomaly alters dramatically the anatomy of the fetal breech, it is likely that the use of surface-rendering reconstruction might have significantly contributed to the diagnosis; in addition, the employment of the multiplanar imaging technique might have allowed for a detailed study of the anatomic relationship between the various elements of the complex malformation.

The management of SNS is basically tailored on the different anomalies present in each case. It aims primarily at the restoration of the intestinal function with separation of the neuroenteric fistulas, necessary to avoid fecal contamination of cerebrospinal fluid, if required (this did not apply to the index case for there was no neuroenteric communication in this case). The second issue is the excision of the lipomyelomeningocele, which should ensue as soon as possible, even in asymptomatic neonates, to avoid or reduce irreversible neurological complications (sensory loss, bladder dysfunction, motor loss, foot deformities and leg pain) associated with delayed release of a tethered cord. In our case, the surgical approach consisted of a two-stage procedure: the initial operation, at two months of age, included the excision of the protruding colon segment and the posterior sagittal anorectoplasty; the neurosurgical intervention, performed at three years of age, consisted of the excision of the lipomyelomeningocele and the release of the tethered cord. However, in this case, the moderately severe bladder and intestinal dysfunctions persisted unchanged after the operation.

In conclusion, we have reported the prenatal findings and the post-natal surgical management of a complex form of spinal dysraphism, the split notochord syndrome, featuring a very rare cluster of anomalies

Table 1—Cases of split notochord syndrome associated with dorsal enteric fistula and/or prolapsed natural colostomy reported to date

Author, year	Spinal defect	Congenital colostomy	DEF	Anus	Associated anomalies	Surgery	Status
Akgür <i>et al.</i> , 1998	T10-L5	No	Yes	Incontinent	Meningocele, dorsal enteric diverticulum near to dorsal enteric fistula	Yes	Dead
Alrabeeah <i>et al.</i> , 1988 ^a	Lumbosacral	No	Yes	Imperforate	NO	No	Dead
Asgar <i>et al.</i> , 2002 ^b	Double split (thoracic and lumbar)	Yes	Yes	Normal	Skin-covered meningocele with intestinal loop, enteric fistula and a fully developed lower limb	No	TOP
Bentley and Smith, 1960 ^c	L2-sacrum	No	Yes	Imperforate	NO	No	Dead
Burrows and Sutcliffe, 1968	L2-sacrum	Yes	Yes	Normal	Meningocele, colonic duplication	Yes	Alive
Faris and Crowe, 1975	T10-sacrum	Yes	Yes	Imperforate	Meningocele, lumbosacral mass containing small intestine and stomach	Yes	Dead
Gupta and Deodhar, 1987	L5-sacrum	Yes	Yes	Normal	Meningocele	Yes	Alive
Hoffman <i>et al.</i> , 1993	T10-sacrum	Yes	Yes	Normal	Meningocele, cloacal extrophy	?	Dead
Kannaz <i>et al.</i> , 2002	Lumbosacral	No	Yes	Normal	Dysgenetic <i>corpus callosum</i> , lipomyelomeningocele, gastrointestinal malrotation, wandering spleen, right inguinal hernia	Yes	Alive
Keen and Coplin, 1906	L3-sacrum	No	Yes	Normal	Sacroccygeal teratoma	Yes	Alive
Kiristioglu <i>et al.</i> , 1998	L1-sacrum	Yes	Yes	Imperforate	Meningocele, foreshortened colon	Yes	Alive
Kramer <i>et al.</i> , 1988	T10-sacrum	No	Yes	Imperforate	Meningocele, sacral agenesis	No	Dead
Meller <i>et al.</i> , 1989	Lumbosacral	Yes	Yes	Anteriorly displaced	No	Yes	Alive
Pathak <i>et al.</i> , 1988	Double split (C1-D5; D12-sacrum)	Prolapsed stomach and small intestine	No	Imperforate	Short colon	No	Dead
Rembe ^d	L1-sacrum	Small intestine and blind pouches	Yes	Normal	?	?	?
Singh and Singh, 1982	T10-sacrum	No	Yes	Normal	Hydrocephalus, depressed nasal bridge, ear anomalies	No	Dead
Our case	L4-sacrum	Yes	Yes	Imperforate	Lipomeningocele; tethered cord; rectourethral fistula	Yes	Alive*

DEF, dorsal enteric fistula.

^a one of a series of seven;^b one of a series of four;^c one of a series of five;^d cited by Faris and Crowe, 1975

(SNS, colostomy-like dorsal opening without exposed intestinal mucosa, anal atresia, rectourethral fistula, lipomyelomeningocele). This report demonstrates that SNS can be suspected prenatally whenever a spinal defect is detected together with an unusual gastrointestinal one, the congenital colostomy in our case, though it has to be emphasized that SNS represent extremely rare malformations. The prenatal recognition of this malformative cluster is important for a proper prenatal counselling and *in utero* transport to tertiary referral centres.

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SHORT COMMUNICATION

Early prenatal diagnosis of concordant posterior urethral valves in male monozygotic twins

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The association between monozygotic twins and posterior urethral valves (PUV) in postnatal life has been thoroughly described. In the fetus, the prenatal recognition of PUV is feasible. However, it has been repeatedly reported in singletons but never in monozygotic twins. We describe two cases of early prenatal diagnosis of concordant PUVs in monozygotic twins. In one of the sets, the expression of the disease was different for each twin. Copyright © 2006 John Wiley & Sons, Ltd.

KEY WORDS: posterior urethral valves; prenatal diagnosis; monozygotic twins; prune-belly syndrome

INTRODUCTION

The association between posterior urethral valves (PUV) and monozygotic twins is well established in the pediatric literature, with at least 13 cases reported so far (Morini *et al.*, 2002). In the fetus, both the complete and the incomplete form of PUV can be diagnosed prenatally by ultrasound on the basis of a distended bladder, severe hydronephrosis and oligohydramnios. Although the ultrasound diagnosis of PUV has been reported as early as the 13th week of gestation in singletons (Oga *et al.*, 1994), the prenatal recognition of PUV in twins has yet to be described.

We report the early prenatal diagnosis of concordant PUV in two sets of male monozygotic twins.

CASE REPORTS

Case 1

A 31-year-old Caucasian woman, in her first pregnancy, was referred to our unit at 14 weeks' gestation owing to a suspected case of PUV in one twin of a monozygotic diamniotic pregnancy. Family history was negative for congenital anomalies and for twinning. On ultrasound, we confirmed the monozygosity of the pregnancy, but detected anomalies in the urinary tracts of both twins. In particular, one twin showed complete PUV: an extremely distended and ruptured bladder with urinary ascites (Figure 1A) and severe bilateral hydronephrosis with hyperechoic dysplastic kidneys. In addition, the urinary ascites acted as a contrast medium, enabling us to detect the extremely reduced thickness of the abdominal wall. The other twin was diagnosed with an apparently

less severe form of PUV: moderately distended bladder with evidence of the bladder neck, bladder wall thickening, severely dysplastic hydronephrotic kidneys (Figure 1B), and distended distal urethra (Figure 1C). The amount of amniotic fluid was only moderately reduced in both sacs owing to the early gestational age. No other malformations were detected. Karyotyping by CVS was proposed but was declined by the patient. During the prenatal counseling session, the couple was informed of the type of malformation and of its differential manifestation in each twin. An assessment of fetal renal function by repeated cystocentesis was proposed to be carried out in the least affected twin in order to assess the renal function (Evans *et al.*, 1991; Nicolini and Spelzini, 2001), but the couple refused the procedure and opted for termination of pregnancy. The pathology report confirmed the prenatal diagnosis of PUV in both twins (Figure 3A,B). The twin with the severest form had developed prune-belly syndrome (PBS) (Figure 3A).

Case 2

A 35-year-old woman, gravida 2,1,0,0, was referred to our unit at 15 weeks of gestation with a suspected case of megacystis in both fetuses of a monozygotic diamniotic pregnancy. On ultrasound, both fetuses showed signs of complete PUVs: both had severely distended bladders with evidence of the bladder neck (Figure 2) and hyperechoic dysplastic kidneys. These findings led us to suspect the development of secondary PBS in both twins. The karyotype, obtained by CVS, showed a 46,XY complement. In this case as well, after a thorough prenatal counseling session, the couple opted for termination of pregnancy. The necropsy confirmed the prenatal diagnosis of PUVs. Both the specimens at birth showed secondary PBS (Figure 3C).

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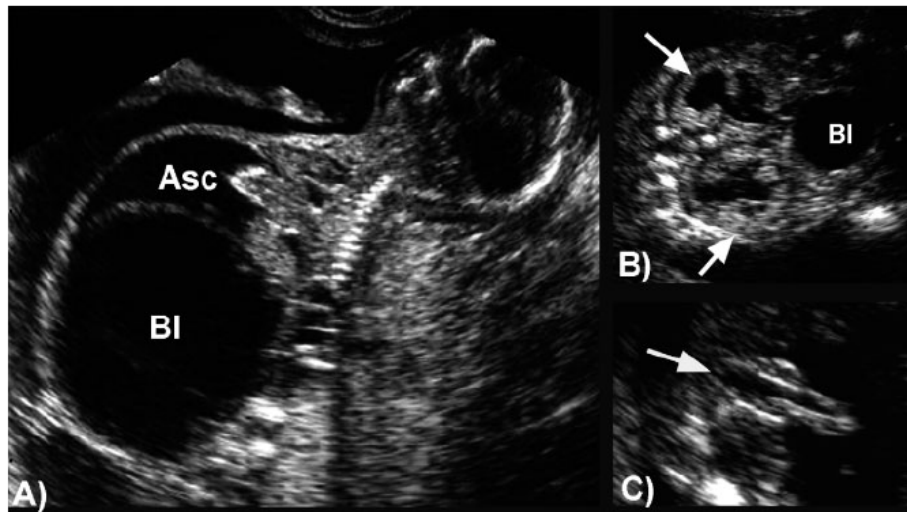


Figure 1—Case 1. (A) shows a longitudinal view of the twin with an extreme form of posterior urethral valves: an extremely distended bladder (BI) and urinary ascites (Asc); note also the thin abdominal wall, consistent with the diagnosis of secondary prune-belly syndrome; (B) shows that the other twin had severely dysplastic and hyperechoic kidneys (arrows), a moderately enlarged bladder (BI) and (C) a distended urethra (arrow)

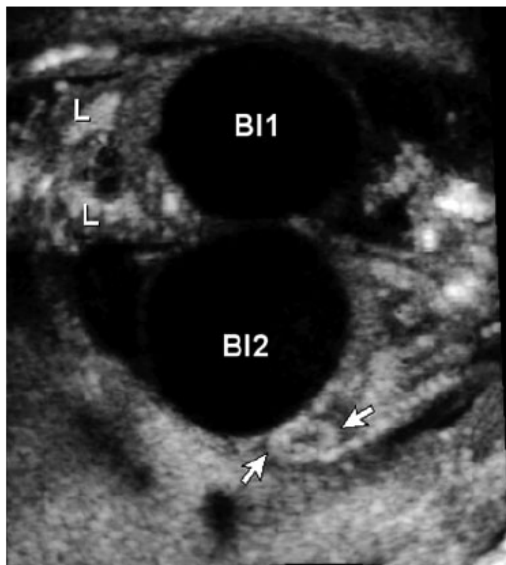


Figure 2—Case 2. Three-dimensional ultrasound—VCI-C rendering. The image shows a coronal view of both fetuses with severely dilated bladders (BI1 and BI2) and severe oligohydramnios. In the upper fetus, the lungs (L) and the heart in between are visible. In the lower fetus, the arrow indicates a hyperechoic dysplastic kidney

DISCUSSION

PUVs represent the most common cause of severe obstructive uropathy in children, with an incidence of

approximately 1 in 1500 to 1 in 8000 boys (Cendron *et al.*, 1994), and they appear to be the cause of about 9% of cases of urinary obstruction in fetuses (Elder, 1997). PUV is a sporadic disorder whose etiology is unknown, though its association with twinning has suggested a genetic cause. It may be related to failure of the complete disintegration of the urogenital membrane, which leaves membranous tissue within the posterior urethra responsible for the obstruction of the bladder outlet (Dinneen *et al.*, 1993).

The prenatal ultrasound diagnosis of PUV is feasible and has already been reported as early as the 13th week of gestation (Sweeney *et al.*, 1981; Dibbins *et al.*, 1985; Turner, 1985; Helin and Persson, 1986; Silver *et al.*, 1990; Oga *et al.*, 1994; Cochat *et al.*, 1996; Rani *et al.*, 1997; Cohen *et al.*, 1998; Yerkes *et al.*, 2001); since the urethral obstruction is not directly detectable through an ultrasound, the diagnosis of PUV depends, in most instances, upon the recognition of sonographic signs of the lower urinary tract obstruction in a male fetus (dilated bladder, hydronephrosis and renal dysplasia (Figures 1 and 2). Additional inconstant findings are represented by a dilated posterior urethra and bladder wall hypertrophy (Glazer *et al.*, 1982; Hayden *et al.*, 1988). The most important prognostic indicator is represented by the presence of a hyperechoic kidney with cortical cysts, which is indicative of severe renal dysplasia (Romero, 1988). The occurrence of renal dysplasia was formerly believed to be uniquely dependent upon the degree and length of the urinary tract obstruction due to misexpression of the Pax-2 gene (Edouga *et al.*, 2001); however, this concept has recently been partially revised and the onset of renal dysplasia is currently considered, at least in some instances, to be primary and synchronous with the

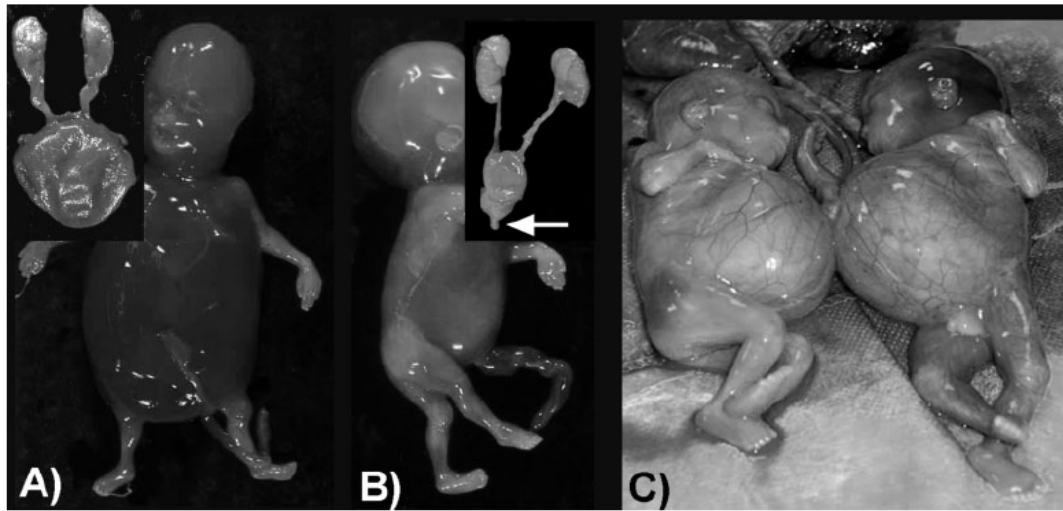


Figure 3—Postmortem examination of the specimens. (A) Case 1, same twin of Figure 1A: the extreme abdominal distention is evident; in the upper left insert, the specimen of the kidneys and the urinary tract with the extremely dilated bladder is shown; (B) Case 1, same twin of Figure 1B: note the lesser abdominal distention in comparison with (A) and the dilated distal urethra (arrow) on the excised specimen (upper right insert); (C) the set of twins of case 2, both showing massive abdominal distention

urinary tract obstruction owing to the misexpression of not only the Pax-2 but also of other genes such as Bcl2 and the transforming growth factor b1 (Winyard *et al.*, 1996; Yoo *et al.*, 2000). Another prognostic factor worth considering is certainly the amount of amniotic fluid: an early onset and severe oligohydramnios is a reliable indicator of impaired renal function and, therefore, such a finding should be part of the prenatal prognostic evaluation. However, it should be underlined that the oligohydramnios sequence starts developing only after 16 weeks of gestation, since until this time the amniotic fluid is mainly produced by filtration through the membranes, and therefore during the first 16 weeks of pregnancy it cannot be considered as a reliable indicator of normal renal function.

The ultrasound differential diagnosis includes other causes of lower urinary tract dilatation, such as massive vesico-ureteral reflux and the megacystis, microcolon intestinal hypoperistalsis (MMIHS) syndrome (Kohler *et al.*, 2004). However, the amount of amniotic fluid is severely decreased in complete PUV, normal or moderately reduced in severe vesico-ureteral reflux and increased in the MMH syndrome. As for the differential diagnosis between PUV and urethral atresia, this is often impossible though the latter instance is a great deal rarer than PUV (Hurwitz *et al.*, 1984).

Whether complete PUV has led to the development of PBS, cryptorchidism and abdominal wall hypoplasia should be detected. In fact, the triad identifying the PBS is represented by the megacystis, cryptorchidism and hypoplastic abdominal wall. However, cryptorchidism can be detected only after the 32nd week of gestation when the testicles are expected to descend in the scrotum in 97% of male fetuses (Achiron *et al.*, 1998), whereas the laxity and thinning of the abdominal wall

musculature are much more difficult to detect; in this case, we could detect it in one fetus of the first set of twins owing to the presence of the urinary ascites which acted as a natural contrast medium (Figure 1A). Therefore, the prenatal diagnosis of PBS may be confidently hypothesized only in the third trimester of pregnancy. The overall prognosis of the condition remains guarded despite the possibility of *in utero* intervention by vesico-amniotic shunting (Gnirs *et al.*, 1988; Perez-Brayfield *et al.*, 2001).

The overall prevalence of structural defects is 1.2–2 times higher in fetuses from twin pregnancies compared to singletons, with most of the excess risk due to increased rates in monochorionic twins (Baldwin, 1994). This is true also for PUV, whose association with twins has repeatedly been reported in postnatal life (Kroovand *et al.*, 1977; Grajewski and Glassberg, 1983; Livne *et al.*, 1983; Romero, 1988). However, despite this association, we were not able to find any case of concordant PUV diagnosed prenatally in twins in a MEDLINE search from 1966 using the key words 'posterior urethral valve, fetus, and twins'. Hence, to our knowledge, the present cases represent the first two cases of concordant PUV in monochorionic twins to be reported prenatally. Of interest is also the different manifestation of the anomaly in each twin of the first set: one presented with a classic PBS secondary to a full-blown form of PUV, whereas the second one presented with an apparently less severe form.

In conclusion, we have documented the early prenatal diagnosis of PUV in two sets of monochorionic twins and described the various manifestations of the disease in the first set of twins. This latter finding should be considered when assessing monochorionic twins with one affected fetus, for the involvement of the other

twin may be subtle and escape ultrasound diagnosis if a detailed examination is not performed.

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Figure 3 The fetus at birth.

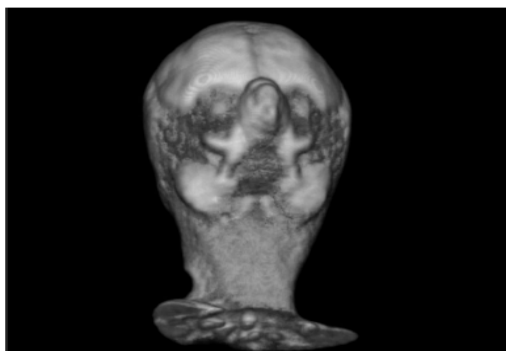


Figure 4 Postnatal three-dimensional reconstructed computed tomographic image showing an otocephalic fetus with mandibular aplasia and ventromedial displacement of the low-lying temporal bones.

The karyotype was normal male 46,XY. Postnatal three-dimensional computed tomography (CT) showed an otocephalic fetus with mandibular aplasia and ventromedial displacement of the low-lying temporal bones (Figure 4).

Otocephaly, a severe malformation of the first and second branchial arches, is characterized by agnathia, microglossia and synotia². Otocephaly has an estimated prevalence of less than one in 70 000 births³. Both hereditary and environmental factors are implicated as causative agents. Otocephaly may occur as an isolated malformation or in association with other anomalies such as cyclopia, holoprosencephaly, neural tube defects, situs inversus totalis, absent adrenals, renal ectopia, horseshoe kidneys, vertebral and rib abnormalities, unilateral lung and congenital heart defects.

We have reported the earliest prenatal diagnosis of otocephaly using prenatal ultrasonography and MRI. Fetal MRI has the advantage of functional evaluation of the fetus, especially in cases of otocephaly, in which multiple anomalies of the brain, heart, skeleton and

internal organs may be found. Reconstructed CT images can reveal the detailed three-dimensional structure of the cranium in otocephaly. In conclusion, fetal ultrasound and MRI can detect otocephaly as early as 15 weeks' gestation, and postnatal reconstructed CT is a powerful instrument for studying congenital anomalies of the skull.

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Massive fetal hemorrhage and fetomaternal alloimmune thrombocytopenia from human platelet antigen 5b incompatibility: an unusual association

We describe a case of fetomaternal alloimmune thrombocytopenia (AIT) to human platelet antigen (HPA) 5b presenting with severe fetal renal hemorrhage followed 1 week later by diffuse subdural hemorrhage.

A 35-year-old Caucasian primigravida was referred to our center at 22 weeks of gestation for suspected severe bilateral hydronephrosis. The woman had unremarkable surgical and medical histories, had not received any blood transfusion nor was receiving any anticoagulant therapy. The targeted ultrasound examination revealed two normal fetal kidneys and no sign of hydronephrosis. However, both kidneys were surrounded by large hypoechoic cyst-like structures with some freely floating specks, indicative of uncoagulated hematomas (Figure 1). There was concurrent significant ascites, possibly of hematic

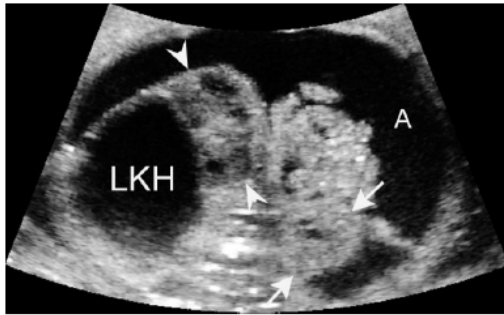


Figure 1 Ultrasound image showing bilateral perirenal hemorrhage (LKH, left kidney hemorrhage). Note how the hemorrhage has dislodged the left kidney from its usual position. A, ascites; arrows, right kidney; arrowheads, left kidney).

origin. No other congenital or acquired anomaly was detected. The patient was counseled about the unusual

nature of the fetus' abdominal problem and agreed to undergo the panel of blood tests that are advisable in cases of fetal hemorrhage. These included serology for parvovirus B19 and cytomegalovirus, and tests to disclose possible AIT. At a follow-up ultrasound examination 5 days after the initial one, in addition to an increase in ascites, the presence of a diffuse subdural, probably hematic, effusion was noted (Figure 2) which was confirmed on magnetic resonance imaging.

All serological tests for prenatal infections proved negative, whereas the assessment of platelet antigens showed parental incompatibility for HPA-5b. Maternal anti-HPA-5b antibodies were detected with a high titer ($> 1:128$), whereas no antibodies against any other platelet glycoproteins were found. Cross-matching maternal and paternal blood was positive. A definitive diagnosis of fetomaternal HPA-5b alloimmunization was established. The peak systolic velocity of the middle cerebral artery was on the 95th centile for gestational age, which could be consistent with the presence of mild

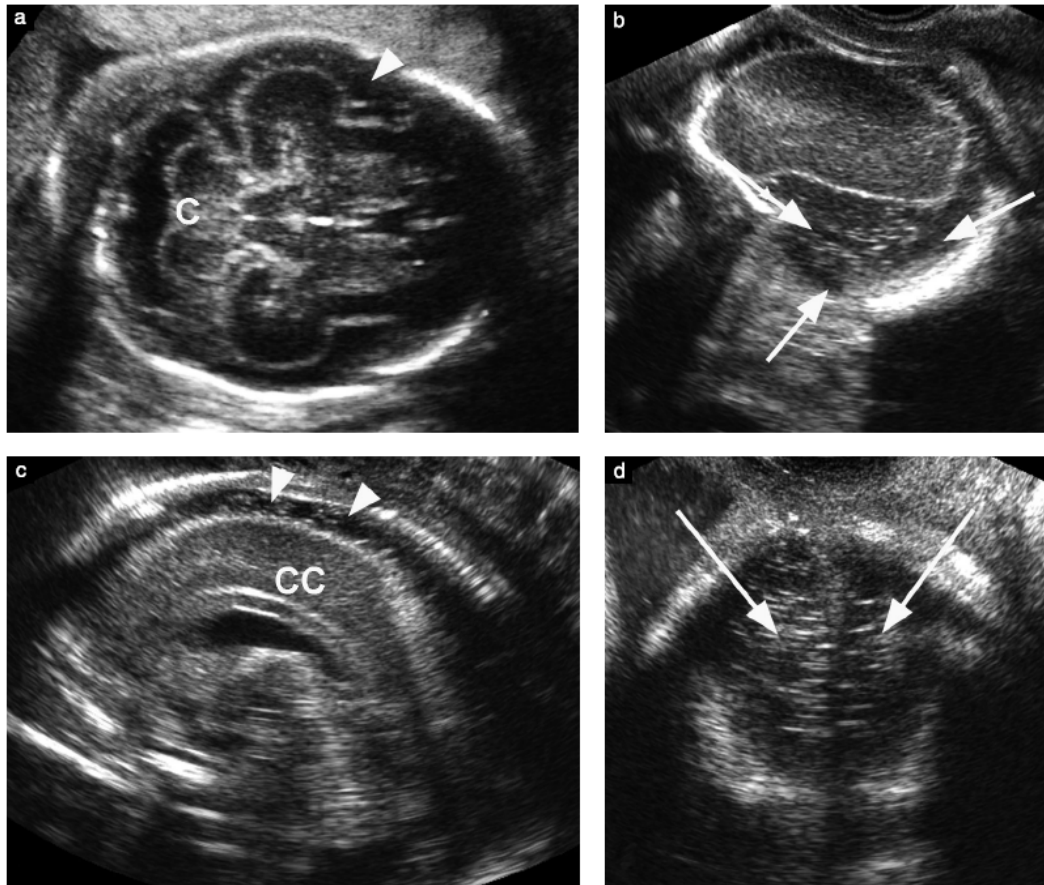


Figure 2 Ultrasound images in different planes showing the subdural hemorrhage (arrowheads and arrows) at different levels: (a) axial view; (b) parasagittal view of the insula/sylvian fossa; (c) midsagittal view; and (d) coronal view of the frontal subdural spaces. At all the sites, visible speckles of blood (arrows and arrowheads) were seen freely moving in the subdural spaces. C, cerebellum; CC, corpus callosum.

post-hemorrhagic anemia¹. A cordocentesis to ascertain fetal platelet and red blood cell counts was proposed, but the patient declined and opted for termination of pregnancy. This was performed by prostaglandin administration via vaginal suppositories at another institution closer to the patient's residence. The necropsy report confirmed the presence of bilateral perirenal and diffuse subdural hemorrhage. The ascites was blood tinged. No other anomalies were found.

AIT results from maternal immunization against fetal platelet antigens of paternal origin. Maternal exposure to these antigens during pregnancy can lead to production of various classes of immunoglobulins: the IgG antibodies are small enough to cross the placental barrier and enter the fetal circulation, causing severe thrombocytopenia. Unlike hemolytic disease of the newborn, AIT can also occur in the first pregnancy if there is parental incompatibility. According to the time of onset, the disease can be referred to as fetomaternal or neonatal AIT. The former often leads to severe hemorrhage and even intrauterine death during the second trimester, whereas the latter represents the most common cause of severe thrombocytopenia in neonates².

To date, five biallelic HPA alloantigen systems have been found to be more frequently associated with severe AIT; they are inherited autosomally and in each codominant antigen pair the more common antigen is designated 'a' and the rarer antigen is designated 'b'. HPA-5 is the biallelic system expressed on the platelet glycoprotein (GP) Ia/IIa. The HPA-5a/b polymorphism is based on a single base variation at position 505 on the *GP Ia* gene³. Among the various platelet antigens involved in AIT, immunization against HPA-1a (P1^{A1}) is by far the most common cause of severe fetal and neonatal thrombocytopenia among individuals of Caucasian origin, accounting for 80–85% of cases^{4–6}. Anti HPA-5b (Br) accounts for 10–15% of AIT cases^{3,4–6}, and the remaining 5% are due to multiple antigen alloimmunization.

In the neonate, HPA-5b alloimmunization has significantly less severe manifestations than HPA-1a alloimmunization, with a reported incidence of intracranial hemorrhage of 0–8% and 14–30% respectively^{5–7}. In pregnancy, the diagnosis of AIT usually follows the detection of massive fetal hemorrhage leading to severe anemia or death, and is due to HPA-1a in the overwhelming majority of cases^{2,6}; to the best of our knowledge, severe hemorrhage due to HPA-5b alloimmunization has not previously been reported in the fetus.

Another peculiarity of this report regards the sites of the fetal hemorrhage, namely the kidneys and subdural spaces. In particular, the latter site of hemorrhage is uncommon for AIT, the most common site of cerebral hemorrhage being intraparenchymal⁶. We do not know why the hemorrhage was subdural in this case; it may be speculated that this rare location may be related somehow to the type of antigen involved in the immunization, although reports addressing the types of lesion related

to HPA-5b AIT in the neonate confirm the preferential intraparenchymal location⁶.

We acknowledge that a limitation of this report is that we do not have direct evidence of the presence of thrombocytopenia as the couple declined cordocentesis. However, the high anti-HPA-5b titer (>1:128) and the concurrent presence of massive fetal hemorrhage in two places make the causal relationship very likely. In fact, at least in some reports, raised anti-HPA-5b titers (>1:32 or ≥1:64) have been associated with a higher risk of neonatal thrombocytopenia⁸ and, furthermore, in the case of anti-HPA-1a AIT, the maternal antibody titer has been shown to be predictive of fetal thrombocytopenia⁹.

A thorny issue is the therapy of choice for AIT. At least when directed against HPA-1a, this condition progressively worsens if untreated and leads, with increasing frequency, to severe fetal hemorrhage; spontaneous regression has never been reported. Therefore, once diagnosed, AIT should be treated. However, the various therapies proposed so far (weekly high-dose intravenous immunoglobulins alone or with steroids, fetal platelet transfusions with antigen-negative platelets) have yielded controversial results², acknowledged by a recent report in the Cochrane Library¹⁰. Likewise, the suggested employment of Cesarean section as a means of preventing perinatal intracranial hemorrhage has yet to be supported by conclusive data¹⁰.

In conclusion, we have described the prenatal diagnosis of massive fetal multiorgan hemorrhage due to fetomaternal AIT against the HPA-5b antigen, which has not previously been related to such severe lesions in the fetus.

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Diagnosis of femoral hypoplasia–unusual facies syndrome in the fetus

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KEYWORDS: cleft lip; femoral focal hypoplasia; prenatal diagnosis; ultrasound; unusual facies

ABSTRACT

Femoral hypoplasia–unusual facies syndrome (FHUFS) is a rare condition characterized by a variable degree of unilateral or bilateral femoral hypoplasia associated with facial clefting and other minor malformations. The prenatal diagnosis of this condition is possible, but so far has been reported prospectively in only two cases. We review all cases of FHUFS reported in the literature and also describe three cases detected prenatally in the mid-trimester, underlining the variable expression of the syndrome. The reported association with maternal diabetes mellitus and differential diagnosis with other syndromes characterized by femoral hypoplasia are also discussed. Copyright © 2007 ISUOG. Published by John Wiley & Sons, Ltd.

CASE REPORTS

Case 1

A 30-year-old primigravida was referred to our unit at 21 weeks' gestation because of a suspicion of severe bilateral femoral hypoplasia in her male fetus. The family history was negative for congenital anomalies. The patient did not have insulin-dependent diabetes mellitus. Ultrasound examination showed fetal biometry consistent with a gestational age of 21 weeks, with the exception of the femora, which appeared severely hypoplastic. The left femur was completely absent whereas the right one measured only a few millimeters (Figure 1a and b). Ultrasound imaging of the fetal face revealed the presence of unilateral right cleft lip and palate (Figure 1d). Unilateral right renal agenesis was also observed. In the counseling session, the couple was informed of the putative diagnosis and outcome of femoral hypoplasia–unusual facies syndrome (FHUFS), and the possible management options.

After counseling, the couple opted for termination of the pregnancy. The diagnosis of FHUFS was confirmed at autopsy. In particular, severe bilateral and asymmetric femoral hypoplasia (Figure 1c), unilateral cleft lip/palate (Figure 1e) and unilateral renal agenesis were found. In addition, the face showed the typical features of FHUFS: a long philtrum, thin upper lip, moderate micrognathia and low-set ears (Figure 1e). Other features consistent with the diagnosis of FHUFS found at autopsy included a short neck and a pelvis with vertically orientated iliac blades.

Case 2

A 28-year-old obese woman (gravida 2, para 1) with insulin-dependent diabetes mellitus was referred to our unit at 21 weeks' gestation because of an abnormal second-trimester anomaly scan. The family history was unremarkable, with no consanguinity reported. On ultrasound examination, severe hypoplasia of the left femur was observed (Figure 2a), whereas the contralateral femoral shaft appeared unremarkable, with length in the normal range¹. Examination of the fetal face revealed severe micrognathia (Figure 2c) and the ears appeared low set. No other abnormalities were observed. A diagnosis of FHUFS was made. After counseling, the couple opted for termination of the pregnancy. At autopsy, severe unilateral hypoplasia of the left femur, severe micrognathia and low-set ears were confirmed (Figure 2b and d). Also noted were a short nose with a broad tip, a thin upper lip and a cleft palate (Figure 2d, inset), confirming the diagnosis of FHUFS.

Case 3

A 24-year-old primigravida was referred to our unit at 13 weeks' gestation after the detection of abnormal lower limbs at the nuchal translucency scan. There was no

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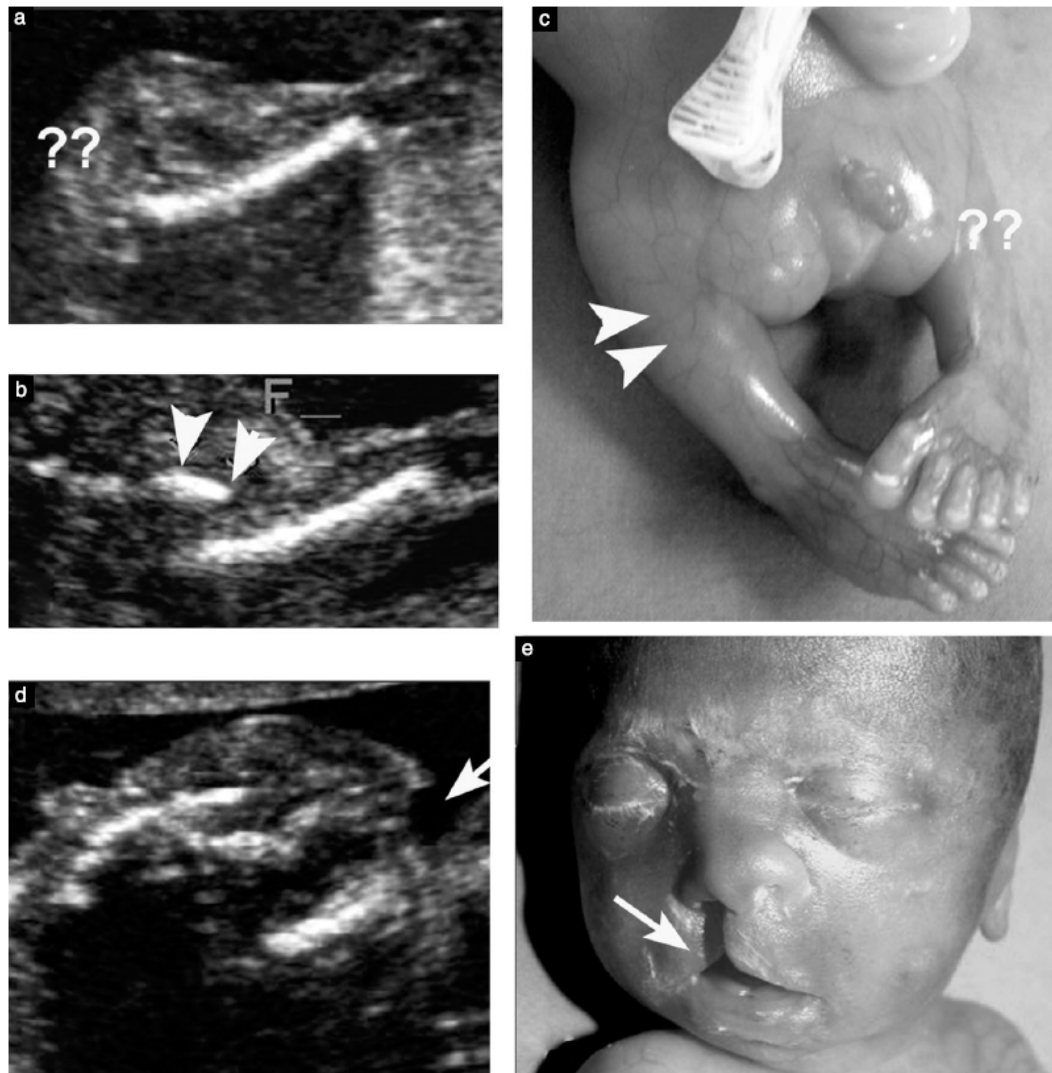


Figure 1 Case 1 at 21 weeks' gestation. (a, b) Ultrasound images showing symmetrical femoral hypoplasia; the femur is completely absent on the left side (a; ??) and severely hypoplastic on the right (b; arrowheads and F). (c) Confirmation of the diagnosis at necropsy; note also the talipes. (d) Axial view of the fetal head showing the wide cleft of lip and palate (arrow). (e) Confirmation of the diagnosis at necropsy; note also the additional subtle facial features, including upslanting palpebral fissures, long philtrum and thin upper lip.

history of either insulin-dependent diabetes mellitus or congenital anomalies. Ultrasound examination revealed a nuchal translucency thickness consistent with gestational age and confirmed the presence of symmetrically hypoplastic femora with normal tibiae and an abnormal left foot (Figure 3a and b). Transvaginal ultrasound examination further confirmed these findings and, in addition, suggested the presence of moderate micrognathia (Figure 3b). Termination was carried out in such a way that an intact specimen was obtained for post-mortem examination. The pathologist confirmed the

diagnosis of FHUFS and described severely hypoplastic femora, an abnormal left foot and moderate micrognathia (Figure 3c). No additional facial dysmorphisms or other associated anomalies were detected at necropsy at such an early gestational age, but the pathologist felt confident with the diagnosis of FHUFS.

DISCUSSION

Femoral–facial syndrome, or FHUFS, is a rare syndrome, described for the first time in 1975². The pathogenesis of

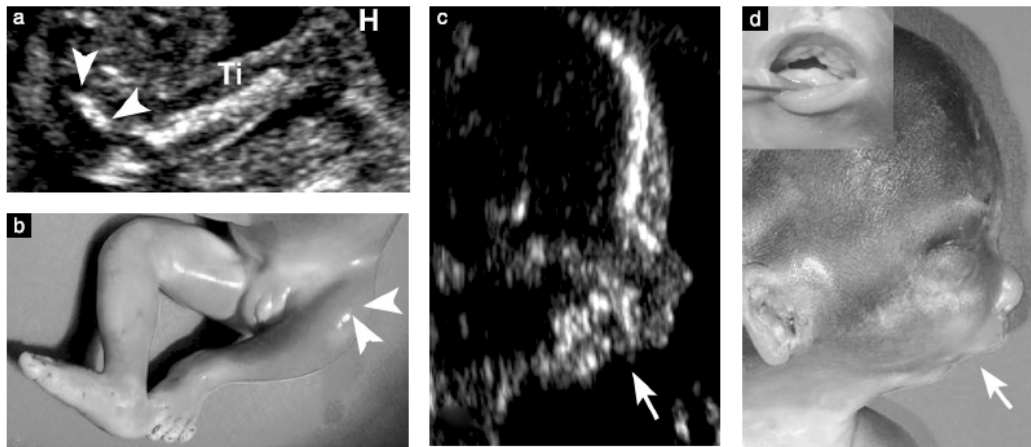


Figure 2 Case 2 at 21 weeks' gestation. (a) Ultrasound image showing severe asymmetrical femoral hypoplasia; note the severely shortened femoral shaft (arrowheads) and the normal tibia (Ti) and foot (H, heel). (b) Confirmation of the diagnosis at necropsy. (c) Sagittal view of the facial profile, showing severe micrognathia (arrow). (d) Confirmation of the diagnosis at necropsy (arrow). Note also the low-set ears, long philtrum and thin upper lip. The associated cleft palate is shown in the inset.

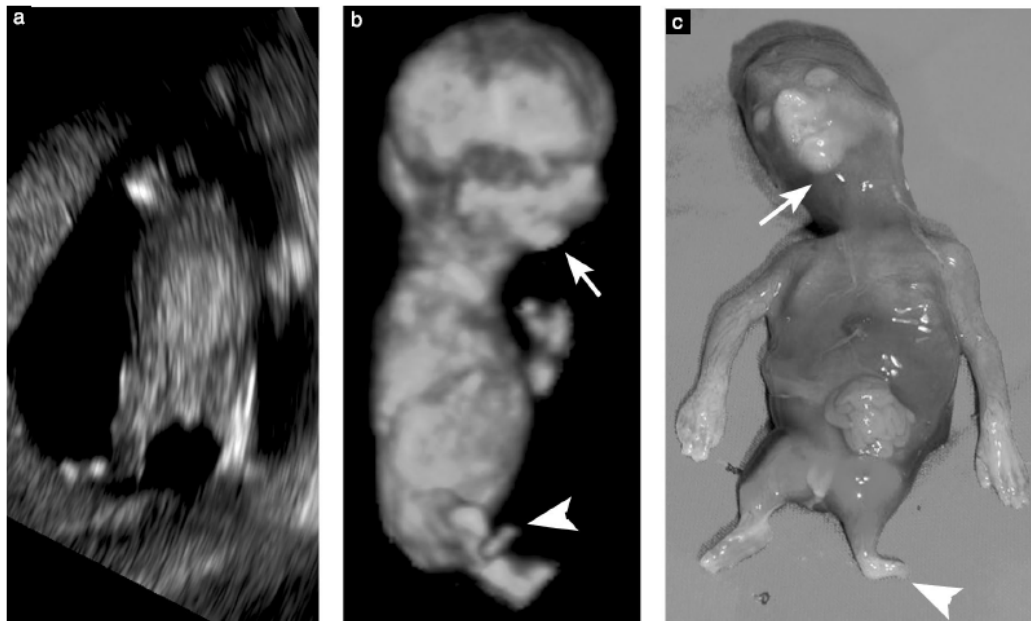


Figure 3 Case 3 at 13 weeks' gestation. (a) Two-dimensional ultrasound image of the severely hypoplastic femora. (b) Three-dimensional surface-rendered image of the whole fetus demonstrating the severe bilateral femoral defect and the abnormal left foot (arrowhead); moderate micrognathia (arrow) was also suspected. (c) Confirmation of the diagnosis at necropsy; the arrow indicates the moderate micrognathia and the arrowhead points to the abnormal foot. The abdominal wall lesion with bowel herniation was traumatic and occurred during expulsion of the fetus following rupture of the membranes.

the syndrome is unknown, although an association with maternal insulin-dependent diabetes is well documented², as are the large number of similarities with caudal regression syndrome, another condition frequently seen in infants of diabetic mothers. Most cases of FHUFS

have been sporadic, although a few cases of Mendelian inheritance have been reported.

The key features of this condition are bilateral and often asymmetric focal femoral hypoplasia and facial dysmorphism, the latter ranging from evident

Table 1 Time of diagnosis and ultrasound and pathological findings in fetuses diagnosed with femoral hypoplasia—unusual facies syndrome

Reference	Ultrasound findings					Time of diagnosis
	GA (weeks)	Femoral hypoplasia or aplasia	Hypoplasia or aplasia of other bones	Facial signs	Other anomalies	
Tadmor <i>et al.</i> ⁴	32	+	+	CLP	-	Prospective
Robinow <i>et al.</i> ⁵	25	+	-	Micrognathia	-	Prospective
Campbell and Vujanic ⁶	19	+	-	-	-	Necropsy
Urban <i>et al.</i> ⁷	21	+	+	-	-	Necropsy
Gillerox <i>et al.</i> ³	25	+	-	-	-	Necropsy
Filly <i>et al.</i> ⁸	20	+	-	-	Clubfoot	Necropsy
This study						
Case 1	21	+	-	CLP, micrognathia	Unilateral renal agenesis	Prospective
Case 2	21	+	-	Micrognathia	-	Prospective
Case 3	13	+	-	Micrognathia?	-	Prospective

ACC, agenesis of the corpus callosum; CLP, cleft lip/palate; GA, gestational age.

micrognathia/cleft lip and palate to more subtle features such as upslanting palpebral fissures, short nose with broad tip, long philtrum, thin upper lip, maxillary asymmetry and isolated cleft palate (Figures 1e and 2d). A number of additional malformations have been described occasionally in neonates with FHUFS, including lumbar spine and pelvic abnormalities, absence or hypoplasia of the fibulae, talipes and genitourinary anomalies². Furthermore, severe central nervous system (CNS) malformations have been reported in neonates with fatal FHUFS, including brain heterotopia, agenesis of the corpus callosum and ventriculomegaly³. It can therefore be said that there is moderate variation in the phenotypic expression of FHUFS, with occasional reporting of associated CNS and spinal anomalies.

Prenatal ultrasound diagnosis of FHUFS is feasible given that its most consistently reported findings, namely femoral hypoplasia and major facial anomalies, can be recognized *in utero*. However, a prospective diagnosis of FHUFS, based upon the concurrent detection of femoral and facial anomalies, has been reported in very few cases^{4,5}; in most published reports the diagnosis was made at autopsy following prenatal diagnosis of the focal femoral defect only (Table 1)^{3,6-8}. This is probably because prenatal recognition of the femoral defect, unlike facial anomalies, is relatively straightforward on ultrasound examination. In addition, it should be noted that it is the facial dysmorphisms, although subtle in some cases, that need be recognized to support a prospective diagnosis of FHUFS in the fetus. In fact, the focal femoral defect can occur as an isolated anomaly or represent one of multiple abnormalities characteristic of a number of rare disorders, such as caudal regression sequence, camptomelic dysplasia, Antley-Bixler syndrome and kyphomelic dysplasia⁹. The differential diagnosis of these pathologic entities may present some difficulties. However, in focal femoral hypoplasia the skeletal anomaly is not associated with any malformation of the craniofacial area, whereas in FHUFS micrognathia and/or cleftings are frequently present and recognizable *in utero* (Figures 1d and 2c). As for caudal regression sequence, which is also associated with maternal insulin-dependent diabetes and fetal urogenital abnormalities, a major lower spinal defect is usually present at the same time, which is extremely rare in FHUFS¹⁰. Camptomelic dysplasia is characterized by bowed and moderately short (rather than severely hypoplastic) femora and tibiae, scapular hypoplasia, micrognathia and sex reversal in males¹⁰. Antley-Bixler syndrome features multiple synostoses, in addition to bowed long bones^{9,10}. Finally, the autosomal recessive disorder kyphomelic dysplasia is characterized by symmetrically hypoplastic and bowed femora and micrognathia⁹; we consider this to be the only condition that may be difficult to distinguish from FHUFS prenatally.

In view of the differential diagnosis described above, we believe that, for a prospective prenatal diagnosis of

FHUFs, at least one other major anomaly of the skeletal or craniofacial region should be detected in addition to focal femoral hypoplasia. If a third malformation is also found, such as unilateral renal agenesis or a spinal or CNS anomaly, then the diagnosis is virtually certain. Despite this relatively simple diagnostic algorithm, only two prospective diagnoses of FHUFs in the fetus have been described to date (Table 1)^{4,5}. Here we report another three cases detected at 21, 21 and 13 weeks' gestation, in which the facial features were carefully sought following the detection of the femoral defect. This report demonstrates that prenatal diagnosis of FHUFs is feasible with a high degree of accuracy if expert ultrasound examination is performed. To the best of our knowledge, the case detected at 13 weeks' gestation represents the earliest prenatal diagnosis of FHUFs and the first with three-dimensional imaging.

Regarding three-dimensional ultrasound examination, this approach is of no additional value in the diagnosis of femoral hypoplasia, which is easily detected on two-dimensional ultrasonography (Figures 1a and b, 2a and 3a). However, it can be speculated that its use might enhance the diagnostic capability of ultrasound in relation to minor facial dysmorphisms, such as upslanting palpebral fissures and, especially, nasal dysmorphisms including short nose with broad tip, long philtrum and thin upper lip. We were not able to provide evidence to support this hypothesis because the index fetus examined with three-dimensional ultrasonography was at a very early gestational age (13 weeks); at this early age, the fine facial features are not fully developed and thus the capacity of ultrasound to detect these subtle features is less than that at 20 weeks' gestation. However, despite these limitations, moderate micrognathia was apparent on the three-dimensional surface-rendered image (Figure 3b).

Another important consideration is that the development of femoral hypoplasia may also occur relatively late, although this bizarre finding was reported in only one case. In this case, described by Tadmor *et al.*⁴, long bone biometry was unremarkable at scans performed at 19 and 24 weeks' gestation, with facial clefting and shortness of the humeri and femora identified at 32 weeks. At this gestational age, the femur length was described to be at the 50th centile for 24 weeks' gestation. Follow-up examinations at 34 and 37 weeks' gestation showed resumption of normal femoral growth, suggesting that an *in-utero* insult leading to a transient arrest of femoral growth had occurred after the 24th gestational week⁴.

Finally, as far as terminology is concerned, we agree with Tadmor *et al.*⁴ and Urban *et al.*⁷, who suggested that FHUFs should be considered as an association of

malformations rather than a syndrome because of the wide range of anomalies observed (Table 1). Generally, the definition of an association is the idiopathic occurrence of multiple congenital anomalies during blastogenesis¹¹, whereas a syndrome is characterized by a cluster of malformations that are known or causally related¹².

In conclusion, we believe that the diagnosis of FHUFs can be reliably established prenatally if at least one major skeletal or craniofacial anomaly is found in addition to asymmetric femoral hypoplasia. Other associated anomalies amenable to ultrasound diagnosis include major CNS (agenesis of the corpus callosum, hydrocephaly), spinal (hemivertebra) and renal (unilateral agenesis) malformations.

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App. 13

Prenatal Diagnosis of placental chorioangioma: our experience.

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ABSTRACT

Placental chorioangioma is the most common benign tumor of placenta. The relationship of vascularized chorioangiomas to adverse pregnancy outcome is well recognized. We report three cases of placental chorioangioma. Hypervascularization of the lesions in all patients and the immune hydrops with adverse fetal outcome in two cases are the complications of the our mini-series. The ultrasonography and Doppler ultrasonography findings were useful in establishing the prenatal diagnosis and the prognosis.

INTRODUCTION

Placental chorioangioma is the most common benign tumor of placenta, encountered in approximately 1% of all pregnancies; probably its origin is hamartomatous. In the cases of prevalent mixomatous component chorioangioma will appear ipoechogenic and negative to the Color-Doppler; in the cases of prevalent vascular component chorioangioma will appear ipoechogenic and positive to the Color-Doppler. Congestive heart failure could be determined because two possible reasons: 1) intralesional mass circulating sequestration, usually associated with tumors greater than 5 cm in diameter; 2)

tumor's compression on the umbilical cord, when chorioangioma is located near the placental hilus. Cardio-circulating failure, if present, will appear with the hydrops features (1,2,3,4). Other possible complications could be: polyhydramnios, preterm birth, fetal anemia, growth retardation (I.U.G.R), fetal anemia, intrauterine fetal death (1,2,3,4).

CASE 1

A 32-year-old-caucasian woman, II Gravida I Para, was referred to our unit at 18 weeks of gestation. Ultrasonography examination revealed a diamniotic bichorial twin pregnancy. An hyperechogenic area of 41 mm positive to the Color-Doppler, was observed in the placenta of one of the two twins (female twin). This lesion was contiguous to the hilus but didn't modify the umbilical cord Doppler's values. Hydrops, ascitis or cardiac failures were not observed. The placenta of the other twin appeared normal. Amniotic fluid quantity was regular in both of the amniotic sacs. Both fetuses biometry and anatomy were regular. Regular fetal fluximetries were indicative of a normal feto-placental hemodynamic. After about 4 weeks the female twin developed congestive heart failure, hydrops and after two days she died. The other twin was delivered at term after caesarean section performed at 39 weeks of gestation. The male baby weighed 3350 g, with Apgar scores 8 and 9 at 1 and 5 minutes, respectively.

CASE 2

A 28-year-old-caucasian woman, I Gravida, came to our observation at 31 weeks of gestation. Fetal ultrasonography revealed a disomogeneous placental formation, high

positive to the Color-Doppler of approximately 71 x 57 mm. Placental hilus was distant to the lesion. Hydrops, ascitis or cardiac failures were not observed. Fetal anatomy and biometry were regular. Hearth Doppler velocimetry was regular. Amniotic fluid quantity and fetal fluximetries were regular. The patient was evaluated every week. A caesarean section was performed at 35 weeks of gestation because the rapid increase of the chorioangioma's biometry and the initial heart failure. The newborn weighed 3210 g, with Apgar scores 7 and 9 at 1 and 5 minutes, respectively.

CASE 3

A 27-year-caucasian woman, I Gravida, was referred to our unit at 24 weeks of gestation. The transabdominal ultrasound examination showed a vascularized placental hypoechogenic formation. The biometry of the lesion was of 82 x 72 mm. A diffused subcutaneous hydrops, hydrotorax, cardiomegaly, hepatomegaly, polydramnios were present. These signs are secondary to the hearth failure because the presence of the chorioangioma. Fetal biometry was regular for the gestational age. Remaining fetal anatomy was normal. Amniotic fluid was increased with largest vertical pocket of 95mm (n.v. 20-80 mm) and AFI of 31 (n.v. 6-20). Uterine fluximetry was normal as in right trophoblastic invasion. Arterious fetal fluximetry was regular; venosous fetal fluximetry expressed cardiac failure. Fetal complications as polyhydramnios and cardio-circulating failure were responsible of intrauterine death at 30 weeks of gestation. The pathology report confirmed the prenatal diagnosis of placental chorioangioma.

DISCUSSION

Placental chorioangioma is the most common benign tumor of placenta, encountered in approximately 1% of all pregnancies. (1) Often the tumors have a microscopic volume. The incidence of chorioangiomas enough large to determine an alteration of the fetal cardio-circulating dynamic and which ultrasonographic diagnosis is possible, was approximately 1/8000- 1/50000 pregnancies and 1/3500 to 1/9000 births (1,5,6,7). We know two types of chorioangioma: the angiomatous type with numerous blood vessels and the mixomatous type with mesenchymal tissue and without hypervascularization (1). Large placental chorioangiomas have a variable shape and often protrude from the fetal surface of the placenta near the cord insertion; in these tumors were often observed calcifications, necrotic phenomena (1). The sonographic diagnosis is often occasional and it could be associated to an increase of the alpha-feto protein in the amniotic fluid and in the maternal serum. MRI could add clearness to the sonographic feature (9). Sonographic diagnosis is based on the detection of a circumscribed solid mass (hyperechogenic or hypoechogenic) or a complex mass. Most of the tumors are located near the cord placental insertion (1). Jauniaux et al propose that a high echogenicity of the angiomatous lesion is associated to a better prognosis related with the fibrotic degeneration of the tumor (5). Some authors report case of spontaneous tumor infarction with a decreasing of the volume and the echogenicity (10); other authors didn't find correlation between volume and echogenicity changes and the intramural tumor flux (11,12). The 3D-power doppler analysis showed a reduction of the vascular density after a spontaneous infarction of a big lesion without change of typical ultrasonography

features. This feature was probably determined by thrombotic degenerations as reported in the isthologic analysis. Tumor biometry is constant in the second half of pregnancy, even if in our experience there was a rapid increase of the tumors in the second half of pregnancy (case 2 and case 3). Obstetric complications of the chorioangiomas are: polyhydramnios (1,2,3,4), preterm birth, fetal anemia, IUGR (1,2,3,4). The high incidence of the preterm birth is independent by tumor biometry; in all cases of preterm birth could be necessary a analysis of the placenta (8). Polyhydramnios was reported in 33% of the cases (1); its etiology was uncertain although most of the tumors are large and the high vascularization could cause a rise of amniotic fluid because transudation of liquid through the vessels' wall (13). A correlation between polyhydramnios and preterm birth was also reported because the uterine over-distension (5). Amnioreduction was successful reported with a better neonatal outcome. Heart failure and hydrops are complications of the tumor vascularization rather than its volume. This feature is evident in the Case 3. Other complications include toxemia, abruptio placentae, placenta previa (1). A good prognosis could be previewed when a large mass isn't much vascularized (5). This date was also confirmed by other authors in the cases of fibrotic tumor's degeneration. The in utero management and therapy by ablation or an alcohol injection were made with variable results (4,14). Other possible in utero approaches are the fetal transfusion, the tumor vessels ligation (15) and the vessels thermocoagulation. An other intrauterine surgical treatment; is the microembolization with an interstitial laser; this innovative technique was associated with a premature delivery even if the neonatal and maternal outcome were good (16). Differential diagnosis include hematomas (3) (chorionic and subamniotic hematomas), other placental masses (teratomas), partial

hydatidiform mole and maternal placental metastatic tumors (17,18). Frequently the chorioangioma is associated to the twin-pregnancies, the single umbilical artery and the velamentous cord insertion.

The accurate study of placenta structure is a fundamental part of a ultrasonography exam in all the period of pregnancy; a prenatal ultrasonography diagnosis of the chorioangioma is possible in many case (4,8). The potential value of 3D power Doppler in prenatal diagnosis and monitoring of pregnancies complicated by large, vascularized chorioangioma was recently recognized.(12)The strict ultrasonography surveillance is necessary to prevent the unfavourable fetal outcome of the largest tumors as happened in the case 2; the tumor's volume evaluation and the vascularization of the mass by Doppler are high useful to monitorize the possible fetal complications. The volume's increase of the mass and the possible effects of the chorioangioma on fetoplacental hemodynamic, fetal growth and amniotic fluid quantity are well recognized by ultrasonography. When the patient decline the fetal invasive therapy, whose complications are high again, the recognition of the ultrasonography features of an high vascularization's grade of the mass and the onset of the complications are useful parameters which offer to the obstetrics the choice of caesarean section in consideration of the fetal pulmonary maturity

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App.14

PRENATAL DIAGNOSIS OF ARTHROGRYPOSIS

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Abstract

Arthrogryposis, is an uncommon congenital syndrome clinically characterized by the occurrence of joint contractures of variable etiology that start prenatally. This disease may result from no apparent hereditary causes (neuropathic, for example) or may be the result of hereditary factors (myopathic form, for example). Ultrasound diagnosis depends on observation of scant or absent motion of fetal extremities. Prognosis depends on the specific etiology of the contractures. We report 4 cases of prenatal diagnosis of Arthrogryposis.

Introduction

Arthrogryposis is an uncommon congenital syndrome clinically characterized by multiple joint contractures that progressively deform child osteo-skeletal, without other congenital anomalies and with a relatively regular intelligence. When this presentation is in association with other congenital anomalies, it is defined arthrogryposis multiplex congenita (AMC) that develops in utero and may be related to any of a heterogeneous group of disorders (table1). The incidence of arthrogryposis is in relation to the reported incidence of AMC and that varies from one on 3000-10 000 live births. Over 150 different etiologies have been described but most cases show a neurogenic form of arthrogryposis; less often it is a primary muscle disease. It is possible a connection with

autosomal trisomy, especially with 18 trisomy, about 4%. About pathological aspects all forms are thought to result from fetal akinesia and the causes of this may be extrinsic factors (oligohydramnios, amniotic bands or uterine fibroids) or primary condition of CNS and PNS.

We report 4 cases of prenatal diagnosis of Arthrogyrosis and discussing counselling and management of this variable disease.

Clinical series

Case1

A 35 years-old Caucasian woman, gravida III, was referred to our unit at 24 weeks of gestation. The ultrasonography examination of the fetal head didn't reveal the cavum septum pellucidum as in cases of corpus callosum hypoplasia/agenesis; the third ventricle was turned up and it was slightly expanded; also bilateral ventriculomegaly was noted. During the examination we didn't observe fetal movements and we noted fixed extended knees. Bilateral clubfoot were present. Fetus biometry and the remaining anatomy were seen as regular. A diagnosis of arthrogyrosis was suspected. Other anomalies were not observed.

Case2

A 29 years-old Caucasian woman, gravida I, was referred to our unit at 20 week of gestation for an ultrasonography control. The trans-abdominal ultrasound examination showed a fetus affected by distal arthrogyrosis. Bilateral clubfoot were present and bothe hands were constantly closed; the movements of other joints were regular.

Subcutaneous edema of the neck, thorax and abdomen were observed. Fetal biometry and amniotic fluid quantity were regular. Regular fetal fluximetries were indicative of a normal feto-placental hemodynamics. Other anomalies were not observed. The final diagnosis was distal arthrogryposis. The infant presented also inguinal hernia.

Case 3

A 31 years-old Caucasian woman, gravida III, was referred to our unit at 33 weeks of gestation because previous fetus affected by bilateral clubfoot and arthrogryposis. Ultrasound examination showed a pregnancy in evolution complicated by arthrogryposis. Fetus showed constantly lower limbs in the typical position as “rifle barrel” (constant extension of legs and thighs). Both feet were in a regular position but plantar images showed a good curvature. Upper limbs showed a constant position in flexion and hands were constantly closed (half-closed). Fetal biometry was reduced of 12 days. Amniotic fluid quantity was regular. Other anomalies were not observed. From the situation we suspected arthrogryposis.

Case 4

A Caucasian woman was referred to our unit at 28 week of gestation for a routine examination. The ultrasound examination showed a fetus affected by arthrogryposis. During all the time there were poor fetal rolling movements. Thighs were fixed in extension (rifle barrel). No movements of knees and upper limbs. Left hand was in forced flexion while right hand was fixed with forearm fixed on thorax. Stomach and external genitalia were not observed. Fetal biometry was bigger than 9 days; Amniotic fluid was increased with largest vertical pocket of 111mm and 158 in cm².

Discussion and conclusion

Arthrogryposis deforms joints determining non progressive contractures caused by the shortening of the flexor muscles. These joints contractures (distant segments of the limbs are the most frequently affected) are typical of this disease and can be monolateral or bilateral. Arthrogriposis is an uncommon congenital disease characterized by multiple joint contractures that deform child osteo-skeletal apparatus, encountered in approximately one on 3000-10 000 live births. Its prenatal echographic diagnosis is quite difficult. Usually these syndromes are discovered in III trimester, even if prenatal diagnosis of severe forms is possible in II trimester. Echographic diagnosis is possible only after a long time that interested limbs maintained an anomalous position. It is always needed to practise fetal karyotype and cardio ultrasonography. Some forms are particularly severe, other have only poor joints movements. It is very important a particular study of the outcome (autopsy after abortus or tissutal biopsy in live births) and a genetic advising to characterize the type of arthrogriposis and to estimate the recidivous risk.

In case of precocious prenatal diagnosis it is necessary to offer to parents the possibility to stop pregnancy (TOP). In the remaining cases it is indicated an orthopaedic examination with precocious physiotherapy that, in the first months, can give an important improvement. Surgical management can be requested later to line up ankylosis corner but the movement is rarely improved.

Ultrasound markers derived from an anomalous position of limbs with evidence of multiplex joints contractures. Lower limbs can be fixed in extension, in crossed-limb

pattern or always flexed. The foot is usually twisted in equine variety. Upper limbs are typically internally rotated at the shoulders that appear in adduction, extended at the elbows, and flexed and deviated ulnarly at the wrists. Hands are usually closed as fist. Sometimes hips are in luxation and can be slightly flexed. Knees are extended and often there is bilateral pes equinovarus. At the end we can see a scoliotic spine and an hypoplasia of the leg muscles. In the severe forms fetus is still. Frequent is polihydramnios and CNS anomalies recur in 10% of cases (hydrocephalus, poroencephalia, agenesis of corpus callosum, microcephalia, Dandy-Walker syndrome, etc.).

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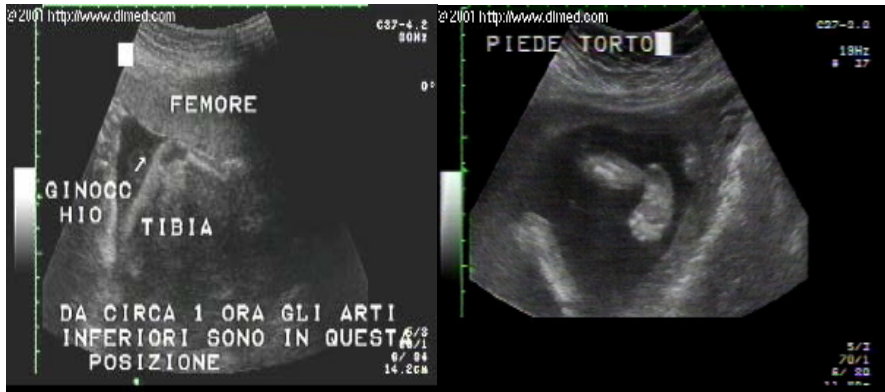
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Table 1	Anomalies associated with arthrogryosis multiplex congenita
<i>Organ system</i>	<i>Abnormality</i>
Maxillofacial	Craniosynostosis, mandibulofacial dysostosis, Micrognathia, macrostomia, decreased TMJ mobility, Microphthalmia, Facial diplegia (Moebius' syndrome), Klepper-Feil syndrome, Low set ears, high arched palate, cleft palate, Loss of supraglottic tone, deficient laryngeal musculature Poor gag reflex, poor suck reflex, dysphagia, esophageal dysfunction
Shoulder	Sprengel's deformity
CNS and spine	Cortical brain atrophy, microgyra, Hydrocephalus, Loss of spinal anterior horn cells, Vertebral anomalies, spina bifida, neural agenesis, scoliosis, kyphosis, Myelomeningocele
Cardiovascular system	Persistent ductus arteriosus Aortic stenosis Coarctation of the aorta
Respiratory system	Tracheoesophageal fistula Hypoplastic lungs Restrictive lung disease from scoliosis Aspiration pneumonia chronic lung disease
Gastrointestinal system	Esophageal dysfunction Gastroesophageal reflux
Genitourinary system	Fusion or absence of the kidneys Renal calculi Cryptorchidism, scrotal and labial defects Absence of the vagina/uterus Hypospadias
Abdomen	Inguinal hernias
Extremities	Absence of patellas Syndactyly Constriction bands Clinodactyly Muscle atrophy



Case 1 and 2



Case 3 and 4

PLACENTA, PRECLAMPSIA and INFECTION

Congenital toxoplasmosis causes blindness and mental retardation. In the United States, up to 85% of women of childbearing age are susceptible to infection with the *Toxoplasma gondii* parasite, which causes toxoplasmosis. Up to 50% of *T. gondii* infections are transmitted by ingesting undercooked meat, making toxoplasmosis one of the most clinically significant food-borne diseases in pregnant women. *Toxoplasma* infection is usually asymptomatic or induces mild, nonspecific symptoms. Primary infection of pregnant women with the parasite *Toxoplasma gondii* results in infections of the unborn by transplacental transmission in about 50% of the cases and it may cause serious fetal effects, but it can be treated, thus reducing the risk of fetal malformations. The Italian National Health Service supports screening for toxoplasmosis in pregnancy. When a seroconversion occurs (2–3/1000 pregnancies), the standard treatment is administration of spiramycin (9×10^6 units day until delivery). (1) Spiramycin has an intracellular toxoplasmocidal activity; moreover, like other macrolides, it inhibits protein synthesis by binding the large (50S) subunit of bacterial ribosomes, causing the growing polypeptide chain to dissociate from the ribosome. (2) Some authors had empirically observed that patients treated throughout gestation for toxoplasma infection seldom developed pregnancy-induced hypertension (PIH) and showed that antibiotic treatment during pregnancy can reduce the incidence of PIH, thus opening new perspectives in its prevention and therapy. (3) There are some evidence that infection can play a role in the pathogenesis of PIH [4-5].

The results of Todros et al (3) suggest that long-lasting spiramycin treatment during pregnancy lowers the risk of developing PIH, probably by preventing the onset of infections that could complicate pregnancy. These findings open new perspectives in the prevention of PIH.

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However, larger prospective studies are required to support the hypothesis that infection plays a role in the pathogenesis of some hypertensive disorders of pregnancy and, ultimately, a randomized trial is required to justify antibiotic treatment as a preventive strategy in clinical practice. Starting to this presuppost we now stay evaluating our date bases; between 1995 and 2006, 542 mother-child pairs were counselled in our department for suspected toxoplasmosis. Repetition of serology and reinterpretation of the serological profile (of all previous results) revealed a false-positive rate of 90% obtained in previous IgM tests and a seroconversion rate of 12% (63/542). We are studying the 63 patient with seroconversion receiving spiramycin and 200 low-risk women who did not take any antibiotic during pregnancy. We stay evaluating also the role of this therapy on a possible anemic state in prevention of onset of preeclampsia. Waiting the results we had printed our uncertainty about the benefits of prenatal treatment of toxoplasmosis.

App. 15 Screening for toxoplasmosis in pregnancy.

We declare that we have no conflict of interest.

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Author's reply

Although many of the panellists in our study have extensive experience with older patients in many different settings (eg, emergency, surgery, medicine, oncology, rehabilitation, and physical therapy), none was specifically a geriatrician, as Marianne Falconer and Desmond O'Neill point out.

The issue of assigning priority in terms of support for activities of daily living was discussed in great detail. Although perhaps more pervasive among older people, this issue, of course, is not restricted to them. It is of similar concern to almost all acutely ill patients, irrespective of baseline health or abilities. Thus the need to consider debility and provision of support for activities of daily living outside the hospital setting in the event of early discharge is independent of age.

Certainly, the panellists did not imply that support for activities of daily living is not important. However, one of the underlying assumptions, as detailed in our report, was that basic care would exist for those discharged to the community, and that such support would therefore be met in other settings. This tempered the assigned priority, since withdrawal of hospital-based support for activities

of daily living was not, in itself, felt to result in an adverse medical outcome requiring a critical intervention.

In the next phase of the study, we will have the ability to determine whether the panellists' assigned priorities for all critical interventions were reasonably accurate, by means of empirically derived data.

I declare that I have no conflict of interest.

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Patient safety and patient error: the carer's perspective

In their Viewpoint on patient safety and patient error (Jan 13, p 158),¹ Stephen Buetow and Glyn Elwyn mention carers (caregivers) only in their opening paragraph. Although patient error has received some attention in published studies, the relevant activities and experiences of carers have almost totally been ignored. The contribution of carers to health care in the UK has been valued at £57.4 million annually² and medicine-related tasks have been shown to be an integral part of the wider caring role with their own associated carer-burden.³

Carers can undertake a wide range of medicine-related activities, each of which presents its own potential for error:⁴ monitoring supplies and ordering repeat prescriptions; assisting with administration of complex regimens, frequent dosing, and multiple forms of dosage and administration; and "clinical roles", including advising on as-needed and regular medicines and management of side-effects, for which they might have limited access to, and problems interpreting, information.

Carers operate in a partnership with their care-recipient⁵ and oft-

en other carers. When differing perspectives regarding the risks, benefits, and value of medicines arise, this will affect the medicine-related behaviours of carers and care-recipients—eg, dilemmas relating to the sharing or concealment of information or their control. The relationship between the patient and health professional, and issues of confidentiality and autonomy, take on a further dimension when carers are included, since patients and carers have their own independent needs and agendas.

A conceptually valid model or taxonomy of patient error must embody the dimensions and effect of caring.

I declare that I have no conflict of interest.

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Screening for toxoplasmosis in pregnancy

The Syrocot Study Group (Jan 13, p 115)¹ states that it is unclear whether prenatal antitoxoplasma treatment has any benefit. This result needs urgent confirmation since it poses the basis for a radical change in prenatal management.

Although some researchers have raised doubts about the effect of

The printed journal includes an image merely for illustration

Illustration

prenatal treatment for toxoplasma infection,² there has never been a definitive consensus. Nevertheless, mass screening for toxoplasmosis is mandatory in some European countries, including Italy, with a consequent "cost" to both the public and patients. In theory, screening can only be done if there is a reliable and valid test to detect the disease in its preclinical stage and if advantages of interventions are clear.³ But there is evidence that serological screening in pregnancy is not reliable.^{4,5}

Between 1995 and 2006, 542 mother-child pairs were counselled in our department for suspected toxoplasmosis. Repetition of serology and reinterpretation of the serological profile (of all previous results) revealed a false-positive rate of 90% obtained in previous IgM tests and a seroconversion rate of 12% (63/542).

The rationale behind the decision to continue screening was that benefits to the fetus outweigh the consequences of a false-positive result (anxiety, invasive prenatal diagnosis, treatment of uninfected women). However, given the uncertainty about the benefits of prenatal treatment, is it ethically correct to carry on?

We declare that we have no conflict of interest.

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Early child development in developing countries

"Finally I have data to convince my Minister of Finance to invest in early child development" reported the Minister of Women and Child Development, Malawi, at a meeting held at the Institute of Child Health, London, UK, to mark *The Lancet's* series on child development in developing countries (Jan 6-Jan 20).^{1,2}

At the meeting, hosted by the Centre for International Health and Development, presenters explained that more than 200 million children younger than 5 years are not developing to their potential owing to poverty, poor health, and nutrition. Although effective interventions are available, coverage is low. Representatives from WHO, UNICEF, and the World Bank expressed a strong commitment to strengthening programmes and research to move the *Lancet* recommendations forward. Other agencies including UNESCO, the Bernard van Leer and Aga Khan Foundations, and many non-governmental organisations explained how they are putting the recommendations into practice.

The *Lancet* steering group will become the International Child Development Committee. This year, we plan to advocate for early child development programmes through presentations at meetings in Turkey, India, Spain, Venezuela, and Bangladesh and at the Society for Research in Child Development and the Pediatric Academic Societies. We will meet at the Rockefeller Foundation's Bellagio Study and

Conference Center to develop implementation strategies and establish priorities in collaboration with the Child Health and Nutrition Research Initiative. We will provide guidance in assessment of existing programmes, development of new models for delivering services, and integration of child development activities into health and nutrition services. In 2 years, we will report on global progress in early child development programmes.

The International Child Development Committee comprises S Grantham-McGregor, P Engle, M Black, M Cabral de Mello, J Meeks Gardner, B Lozoff, N Ulkuer, T Wachs, S Walker, and M Young. I declare that I have no conflict of interest.

Sally Grantham-McGregor, on behalf of the International Child Development Committee

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Risk factors for adverse outcomes in developing countries

In their child development series article, Susan Walker and colleagues (Jan 13, p 145)¹ cite two of our recent publications:^{2,3} "Two small Brazilian studies suggest an association between incidence of diarrhoea in the first 2 years of life and impaired cognitive performance in later childhood.^{8,9} However, a larger cohort study in Peru with control for

Preeclampsia, pregnancy complications and low concentrations of protein Z.

Inherited thrombophilias are a heterogeneous group of conditions which have been implicated in a variety of pregnancy complications. Evidence is mounting that implicates these inherited disorders in a range of pregnancy outcomes, including recurrent miscarriage, late fetal loss, preeclampsia, abruptio placentae, and intrauterine growth restriction. Although the prevalence of these complications is approximately 8% in the general population, their presence is associated with a significantly increased recurrence risk. The most commonly identified inherited thrombophilias consist of Factor V Leiden and the prothrombin gene mutation G20210A. Rarer inherited thrombophilic conditions include deficiencies of protein S, C and antithrombin. More recently, deficiency of protein Z has been linked to pregnancy complications, including preterm delivery and Preeclampsia. Clinical manifestations often are associated with the presence of more than one inherited thrombophilia, consistent with their multigenic nature. Some, but not all, studies investigating the use of heparin to prevent adverse pregnancy outcome have demonstrated a benefit. Protein Z, a vitamin K-dependent plasma protein, has an important role in the regulation of the coagulation cascade. Protein Z deficiency has been associated with unexplained pregnancy loss and adverse pregnancy outcome in patients with thrombophilia. Several studies were conducted to determine if preeclampsia (PE), small for gestational age (SGA), and fetal demise are associated with changes in maternal plasma concentrations of protein Z. PE, but not SGA or fetal demise, is associated with a

significantly lower maternal median plasma concentration of protein Z than normal pregnancy, and a high rate of protein Z deficiency is observed in patients with PE and fetal demise.

Antenatal administration of heparin to prevent pregnancy complications has shown promise in small studies, but a randomized, placebo-controlled trial is necessary to determine whether heparin administration is beneficial in preventing adverse pregnancy outcome.

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In the evaluation of women with fetal loss, and during the study of levels of protein Z, we found a variant of protein Z for a new mutation of the protein Z gene associated with very low protein levels in women with fetal loss.

App.16

A new mutation within Protein Z gene is associated with very low protein levels in women with fetal loss.

Protein Z levels were measured in 124 women with unexplained fetal losses and compared with those of 104 parous controls. Seven out 124 (5.6%) cases and 5 out 104 (4.8%) controls showed PZ levels under the 5th percentile (0.52 µg/ml). Intron C G-42A gene variant was also investigated and found in 5 (71.4%,) cases and 21 [20.2%, Fisher Exact test $p= 0.008$; OR: 9.88 (95%CI: 2.03-47.1)] controls . In addition, 2 patients with PZ levels under the 2.5 percentile carried a new missense mutation within the exon 8 causing the substitution of a hydrophobic residue (Leu) with a hydrophilic one (Arg) in the trypsin-like serine protease domain.

Gene variant intron C G-42A of protein Z is significantly associated with the occurrence of fetal loss . A new missense sporadic mutation within the exon 8 is described in a patient. with very low protein Z levels.

Protein Z (PZ) is a vitamin K-dependent glycoprotein regulating coagulation cascade because of protein Z-dependent protease inhibitor (1). Reduced circulating levels of PZ have been suggested to play a role in the occurrence of bleeding (2) and deep vein thrombosis (3,4) and in early, (5) as well as late fetal losses, (6) although data about this issue are conflicting (7). We recently (8) found in a cohort of patients with a documented venous thrombosis that the prevalence of PZ levels below the 5.0 (0.52 $\mu\text{g/ml}$) or the 2.5 percentile of controls (0.47 $\mu\text{g/ml}$) was higher in these patients (10.2% and 8.7%, respectively) than in controls (4.1%; OR: 2.7 [95% C.I.: 1.2-7.3] and 2.0%; OR: 4.6 [95% C.I.: 1.5-13.9], respectively). A series of variants naturally occurring within the PZ gene locus were investigated in that setting of patients, and PZ levels were found to be associated with the intron C G-42A and the intron F G79A polymorphisms.

Among a group of women previously (7) investigated for otherwise unexplained fetal losses, 7/124 (5.6%) showed PZ levels under the 5th percentile (i.e. 0.52 $\mu\text{g/ml}$), calculated in a control group formed by 104 parous women with at least one uneventful pregnancy and no fetal loss. Five (4.8%, p: ns) out the 104 controls showed PZ levels under the 5th percentile. Therefore, we decided to investigate, by direct sequencing, whether PZ gene polymorphisms or sporadic mutations could be present in these 7 cases (mean age \pm SD: 33.1 \pm 4.3 yrs) compared to 104 controls (mean age \pm SD : 37 \pm 5.8 yrs).

Informed consent was obtained from all the subjects. Institutional Review Board (IRB) approval was obtained by local Ethics Committee. Women with known causes of

thrombophilia (FV Leiden FII A20210 mutations, natural anticoagulants deficiency or antiphospholipid antibodies) were not considered, as previously described (7) .

Blood samples were collected into vacuum plastic tubes containing 3.8% trisodium citrate and centrifuged at 2,000 g for 15 min to obtain platelet-poor plasma. It was frozen and stored in small aliquots at -70 °C until tested. Protein Z plasma levels were evaluated by means of an enzyme-linked immunosorbant assay (Asserachrom Protein Z, Diagnostica Stago, Asnières, France). DNA was extracted from peripheral blood leukocytes according to standard protocols.[8] Amplifications of regions of PZ gene containing the intron A *g-103a* (intron 1; dbSNP#: rs17880587), intron F *g79a* (intron 6; dbSNP#: rs17882561) and e intron C 42bp (*g-42a*) polymorphisms, were achieved as previously described (8). The significance of the difference in observed genotypes between the groups was tested using the χ^2 -squared analysis . Odds Ratios (ORs) and 95% Confidence Intervals (CI) were calculated. Two (28.6%) cases and 18 (17.3%) controls were heterozygous for the intron A G -103A mutation; AA genotype was not observed in cases, whereas it was present in 3 (2.9%, $p > 0.05$) controls. Genotype AG for the Intron F G79A gene variant was observed in 3 (42.9%) cases and 32 (30.8%, $p > 0.05$) controls; AA genotype was not observed in cases and was present in 5 (4.8%) controls. As far as Intron C G-42A gene variant is concerned, 5 (71.4%,) cases and 21 [20.2%, Fisher Exact test $p = 0.008$; OR: 9.88 (95%CI: 2.03-47.1)] controls showed genotype AG, while no case and 5 (4.8%) controls carried the AA genotype .

In addition, 2 patients carried a new missense mutation within the exon 8 (T14928C, accession number AF 440358) causing the substitution of a Leucine with a Proline in the trypsin-like serine protease domain of the protein. PZ levels in these patients were 0.25

and 0.40 µg/ml, respectively , that means below the 2.5 percentile (0.49 µg/ml) of the controls. It is known that leucine is one of the aminoacids that prefers alpha-helics and that proline disrupts secondary structure since side chain cannot form H-bond (see: www.eng.uci.edu). This residue is highly conserved among different species. Among the controls, no woman carried this mutation. More interestingly, this mutation was not found in 197 patients with deep vein thrombosis or in 197 age-matched controls previously investigated (8). This new sporadic mutation within the PZ gene could explain the very low PZ levels in our patients. In addition, the Intron C G-42A gene variants is associated with the occurrence of pregnancy losses in our small sample of patients. Further studies are needed.

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FETAL LOSS and ECTOPIC PREGNANCY

Ectopic pregnancy continues to be one of the most common gynecologic emergencies and is the leading cause of pregnancy-related first-trimester death in the United States. The rate of ectopic pregnancy continues to rise because of increases in the incidences of its risk factors. However, improved modalities of early diagnosis and treatment have reduced both mortality and morbidity of this condition. Evaluating women with fetal loss, we discovered three women with a particular type of ectopic pregnancy, a cervical pregnancy. Cervical pregnancy is a rare form of ectopic pregnancy in which the blastocyst implants and grows within the cervical canal. Cervical pregnancy occurs in 1% of all extrauterine gestations. The incidence varies from 1/1000 to 1/18 000 pregnancies. This condition has been associated with a high morbidity rate and, in the past, frequently led to hysterectomy as a life-saving procedure. Actually the conservative treatment with preoperative bilateral angiographic uterine artery embolization (BUAE) followed by curettage is considered a valid approach. On the basis of these considerations, we present a study describing a series of 3 cases of viable cervical pregnancy diagnosed by TV-US and treated by means of BUAE and subsequent uterine curettage, underlining the safety of the technique but also its potential risk.

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Case report

Is uterine artery embolization for cervical ectopic pregnancy always safe?

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KEYWORDS:

Cervical pregnancy;
Counseling;
Ectopic pregnancy;
Embolization;
Myoma;
Treatment

Abstract. The study objective was to assess the feasibility and the efficacy of bilateral uterine artery embolization for the treatment of cervical pregnancy. The design was a series of 3 cases of viable cervical pregnancy diagnosed by transvaginal ultrasonography and treated by means of bilateral uterine artery embolization (BUAE) and subsequent uterine curettage. Three women with viable cervical pregnancy underwent BUAE and subsequent uterine curettage in the Department of Obstetric and Gynecology, High Risk Pregnancy Center University "Federico II" of Naples. Measurements included surgical outcomes and preservation of fertility. The treatment was effective in all cases. Two patients resumed normal menstruation about 1 month after the procedure, whereas 1 patient underwent a hysterectomy 2 weeks after embolization because of acute ischemic degeneration of a concomitant myoma. The conservative management of cervical pregnancy with angiographic BUAE is a feasible and effective option, even if subsequent hysterectomy can be required. Counseling is necessary.

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Cervical pregnancy is a rare form of ectopic pregnancy in which the blastocyst implants and grows within the cervical canal. Cervical pregnancy occurs in <1% of all extrauterine gestations.¹ The incidence varies from 1/1000 to 1/18 000 pregnancies,² but recently it was reported to be of 1/8628 pregnancies.³ This condition has been associated with a high morbidity rate and, in the past, frequently led to hysterectomy as a life-saving procedure with consequent shattering effects on future fertility.

Early diagnosis of cervical pregnancy substantially improved with the use of transvaginal ultrasonography (TV-US) and pelvic magnetic resonance imaging (MRI). Moreover, the combination of these 2 techniques permits better definition of disease evolution. Actually the conservative treatment with preoperative bilateral angiographic uterine artery embolization (BUAE) followed by curettage is a considered a valid approach. This strategy is effective both in terms of surgical outcomes, such as control of blood flow and prevention of severe hemorrhage, as well as in preserving fertility.

On the basis of these considerations, the aim of this study is to describe a series of 3 cases of viable cervical pregnancy diagnosed by TV-US and treated by means of BUAE and subsequent uterine curettage, underlining the safety of the technique but also its potential risk.

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Table 1 Summary of patients' characteristics

Patient	Age (yrs)	Spontaneous pregnancy	Potential causative factors	Gestational age at diagnosis	Vital embryo
1	39	Yes	TC	11	Yes
2	40	Yes	Conization	12	Yes
3	43	Yes	Unknown	9	Yes

Description of the series

- T1 Table 1 shows the main characteristics of the 3 cases.
 T2 Table 2 shows the management of cervical pregnancy in our series with related side effects and outcomes.

Case 1

A 39-year old woman (gravida 3, para 0, cesarean sections 2) was admitted to our department with a diagnosis of cervical pregnancy at 9 weeks 5 days gestation. The patient reported a β -thalassemia carrier status. She described her last menstruation as poor vaginal bleeding. At the speculum examination the cervix had a bluish color. TV-US revealed a gestational sac with yolk sac and embryo with positive cardiac activity within the cervical canal having a crown-rump length (CRL) of 32 mm (Figure 1). The patient was hemodynamically stable with a hemoglobin level of 12.8 g/dL.

To better evaluate the uterine cervix and the invasion of trophoblastic tissue, pelvic MRI was also performed. The MRI findings agreed with ultrasonography findings (Figure 2). Conservative management was planned because the patient wanted to preserve her fertility, even if a detailed consent for hysterectomy in case of emergency was required and obtained. She did not receive medical therapy, such as administration of methotrexate. With the patient under general anesthesia, BUAE was performed, as detailed below, to prevent massive hemorrhage, and dilation and suction curettage was performed.

No blood transfusion was required, and the patient was discharged 4 days after the surgical intervention. The patient resumed menstruation 35 days after embolization, although her menses were irregular for the next 6 months.

Case 2

A 40-year-old woman (gravida 2, para 1, cesarean section 1) was referred from another hospital to our department with diagnosis of cervical pregnancy at 11 weeks 4 days gestation. The patient did not report any dilation and curettage, but a history of conization for cervical intraepithelial neoplasia II.

During the recovery in the previous hospital, the woman received 2-cycle methotrexate treatment. In particular, the first methotrexate administration was systemic, whereas the second was intra-amniotic.

TV-US performed on admission in our department revealed a still-viable embryo. The gestational sac was within the cervical canal, the CRL of the embryo was 45 mm, and the fetal heart rate was 155 beats/min. T₂-weighted MRI confirmed the cervical pregnancy with the placenta-like mass and 1 fetus in the uterine cervix.

Because the patient wanted to preserve her fertility, conservative management was planned. Thus the patient was informed of the eventual risks related to the procedure and the possibility to undergo hysterectomy, and informed consent was signed.

Bilateral uterine artery embolization, as detailed below, followed by a vacuum evacuation and curettage of the cervical canal was performed. The estimated blood loss was irrelevant, and the patient did not receive transfusion. She was discharged after 6 days, because we waited for a significant decline of serum β human chorionic growth hormone (hCG) concentration, and normal menstruation resumed 38 days after embolization.

Case 3

A 43-year-old woman (gravida 3, para 0, cesarean section 3) was admitted to our department with a diagnosis of

Table 2 Management of cervical pregnancy in our series, side effects, and outcome

Patient no.	Other therapy before UAE	Preembolization hemorrhage	Arteries embolized	Postembolization surgical treatment	Postembolization hemorrhage	Side effects and follow-up
1	None	None	Bilateral uterine	Dilation and curettage vacuum evacuation	None	None; regular menses
2	MTX	None	Bilateral uterine	Dilation and curettage	None	None; irregular menses for 3 months
3	None	None	Bilateral uterine	Dilation and Curettage + Hysterectomy	None	Status febrile, degenerative leiomyomata, postoperative infertility



Figure 1 Transvaginal ultrasonographic scan of the cervical pregnancy. The gestational sac with the vital embryo is located in the cervical canal (C). The uterine body is empty (U).

cervical pregnancy at 8 weeks 2 days gestation. The patient had an unremarkable medical history (no previous intrauterine procedures or pelvic inflammatory disease). TV-US revealed a gestational sac below the closed internal cervical os and an intramural anterior uterine myoma with a main diameter of 62 mm.

The embryo was viable and had a CRL of 21 mm. The cervical pregnancy and the location and size of leiomyoma were confirmed by MRI. Treatment modalities, together with their benefits and potential risks, were discussed with the patient who signed an informed consent form. Conservative management was planned because patient desired future fertility. Dilatation and suction curettage was performed with the patient under general anesthesia after

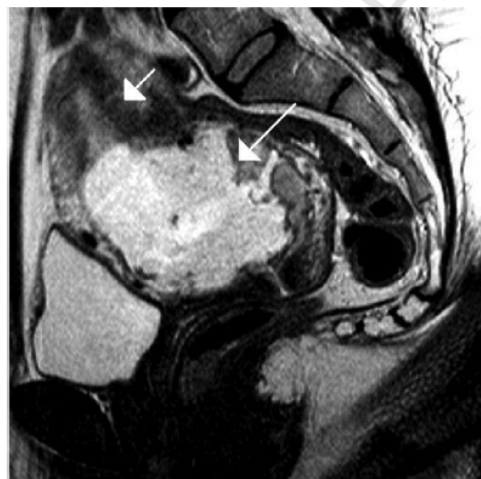


Figure 2 MRI findings include an empty uterus (*short arrow*) and a gestational sac in the cervical canal with trophoblastic invasion (*long arrow*).

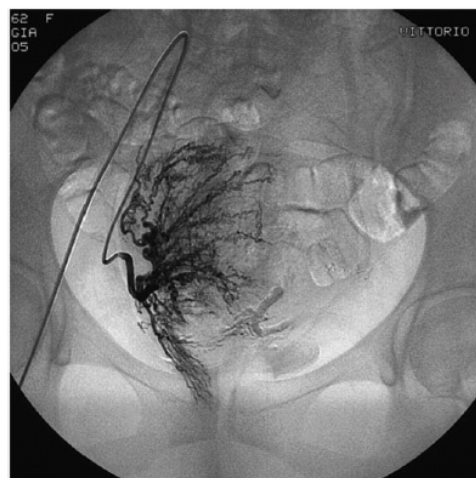


Figure 3 Preambolization angiogram of the right uterine artery. The right uterine artery before embolization showed tortuous arteries in its distal branches dilated because supporting the myoma.

BUAE was performed to prevent hemorrhagic complications. The BUAE is detailed below. Angiography of the right uterine artery before embolization showed tortuous and dilated arteries in the distal branches because they were supporting the myoma (Figure 3).

The estimated blood loss was less than 100 mL, and no transfusion was required. Blood pressure was 115/70 mm Hg. One day after the procedure, a fever developed in the patient. Postoperative ultrasound scanning performed on the fourth day showed a normal cervix but ultrasonographic findings of degenerative myoma. The white blood cell count of $18 \times 10^9/L$ and the cultures indicated a mixed aerobic and anaerobic infection. Antibiotic therapy was started.

Because of the persistence of the febrile status and the worsening of the ultrasonographic findings, we decided to perform a simple hysterectomy. The patient was monitored by daily white blood cell count and hemoglobin measurements and continued to receive antibiotic therapy for another week. Two days after hysterectomy the patient had a normal corporeal temperature, and the white blood cell count returned to the normal range.

Pathologic examination of the removed uterus revealed necrosis, degeneration, and inflammation of myoma tissue caused by the temporary reduction of the uterine flow. The patient was discharged in stable condition after 3 weeks.

Description of the procedure

The BUAE was performed with patients under general anesthesia. The procedure consisted of a percutaneous catheterization of the right femoral artery and both hypogastric

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182 **Figure 4** Postembolization angiogram of the right uterine artery.
183 The vascularity of the mass and myoma was significantly reduced,
184 and the distal arteries are not present.

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arteries via a 5F head hunter-shaped catheter. Selective embolization of the uterine arteries was performed with Spongostan/Surgifoam (gelatin-based absorbable hemostats) for cases 1 and 2 and Embosphere microspheres 500 to 700 μm (acrylic polymer impregnated with gelatin) followed by Bead Block Compressible Microspheres 700 to 900 μm (polyvinyl alcohol, water soluble synthetic resin) for case 3.

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Cessation of vascular uterine circle and complete absence of the myoma vascularization was provided by control angiography (Figure 4). No complication was observed immediately after each procedure.

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202 Discussion

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The use of in vitro fertilization, embryo transfer, and microsurgical techniques, as well as better early ultrasonographic diagnosis, recently increased the rate of ectopic pregnancies. However, cervical pregnancy remains a rare event with an unknown cause, although the frequency of this disease can be influenced by a combination of iatrogenic events, such as previous dilation and curettage, previous cesarean section, previous cervical surgery, or Asherman's syndrome.^{2,4}

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The first ultrasound report of this uncommon type of ectopic pregnancy was reported in 1978 by Raskin.⁵ Even if no maternal death has been reported in the literature since 1953, patients with cervical pregnancy are considered a high-risk group for severe and potentially life-threatening hemorrhage, which may lead to hysterectomy and fertility loss.⁶

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The early diagnosis of cervical pregnancy has improved with the use of ultrasound scanning, leading to a significant decline of complications. In particular ultrasound scanning has been able to differentiate between cervical pregnancy and the cervical stage of miscarriage.⁷ TV-US improves visualization and allows diagnosis by direct visualization of an intracervical ectopic gestational sac or trophoblastic mass. In cases of cervical pregnancy the implantation appears within the cervical canal and is not accompanied by decidual reaction. Therefore the trophoblastic tissue is usually directly attached to the cervical tissue with marked vascularization. A better definition of the uterine cervical invasion by trophoblast is possible with MRI. In fact, the use of MRI improves the evaluation of the tissue's invasion because it allows the visualization of proliferating chorionic villi into the fibromuscular layer.⁶

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Cervical pregnancy is frequently associated with extensive hemorrhage, which, in severe cases, may be stopped only by hysterectomy. However, current treatment options permit effective conservative management in women who want to preserve their fertility. In recent years several fertility-sparing techniques, such as cervical cerclage after curettage,⁴ curettage and packing, local excision and repair, ligation of the cervical branch of the uterine arteries,⁸ systemic or local methotrexate application,⁹⁻¹¹ local intra-sac KCl injections,¹² use of a Foley catheter balloon for compression,^{13,14} and BUAE¹⁵⁻¹⁷ or combination of these,¹⁸ have been proposed.

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Since 1982, methotrexate administration represents the most conservative approach. Furthermore, its effectiveness is reduced in particular conditions, such as serum $\beta\text{-hCG}$ concentration greater than 10 000 IU/L, or a gestational age greater than 9 weeks, presence of fetal activity, and CRL greater than 10 mm.¹⁹ Methotrexate may be also administered as a medical pretreatment to a planned hysterectomy or a very cautious dilation and curettage, because it decreases vascularization of ectopic mass.²⁰

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Another method to decrease the mass vascularization is the BUAE, which stops nutritional support to the trophoblast. The removal of gestational sac and placental tissue is usually followed by bleeding from the vessels supplying the placenta. By selective embolization of 1 or both uterine arteries, the bleeding is usually controlled. This procedure can be performed alone or in association with methotrexate administration. Table 3 shows the main case or series of cases of cervical pregnancy treated with BUAE.

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Bilateral uterine artery embolization for the management of cervical pregnancy was first described in 1990.¹⁵ It was useful to reduce greatly bleeding caused by ectopic pregnancy. With patients under general anesthesia or conscious sedation, a special catheter is introduced through the femoral artery. Catheterization is extended to the internal iliac artery and then to the uterine arteries and is followed by injection of a thrombotic substance (polyvinyl alcohol, gelatin sponge). Bilateral uterine artery embolization was never performed at a gestational age

Table 3 Main case reports of cervical pregnancy

Authors	Date of publication	No. of reported cases	Type of intervention	Outcomes
Nappi et al ¹⁶	1999	1	Methotrexate administration + BUAE + vacuum evacuation and curettage + tamponade with Foley catheter within the cervical canal	Successful hemostasis Preservation of uterus
Yitzhak et al ²⁶	1999	1	Methotrexate intra-arterial administration + selective bilateral hypogastric artery embolization	Successful hemostasis Preservation of uterus
Su et al ²⁷	1999	1	Selective BUAE	Rapid decrease of β -HCG Preservation of uterus
Has et al ²⁴	2001	1	BUAE + methotrexate administration + vacuum evacuation and curettage	Minimal hemorrhage Preservation of uterus Long-term amenorrhea after the procedure
Gui et al ²⁸	2003	1	BUAE + methotrexate administration + BUAE + curettage	Preservation of uterus
Sherer et al ²⁹	2003	1	Methotrexate administration + BUAE + tamponade with Foley catheter within the cervical canal	Complete resolution Preservation of uterus
Takano et al ¹⁷	2004	1	Selective BUAE	Stopping of vaginal bleeding Immediate decrease of β -HCG Preservation of uterus
Vilos et al ³⁰	2005	1	Transfemoral BUAE + resectoscopic evacuation of the gestational product	Minimal maternal morbidity Preservation of uterus
Einarsson et al ³¹	2005	1	Systemic methotrexate + selective uterine embolization	Spontaneous expulsion Vaginal bleeding requiring a tamponade with a Foley catheter
Trambert et al ²³	2005	5	One selective pelvic embolization	Preservation of uterus
		2	Two selective pelvic embolization	Successful hemostasis
		1	One selective pelvic embolization + methotrexate administration	Preservation of uterus One blood transfusion required

later than the seventh week until 1999, when it was first successfully performed at 12 weeks.¹⁶

Although BUAE could be useful in emergency conditions after heavy vaginal bleeding,²¹ we performed BUAE before dilation and curettage to prevent hemorrhage as showed by other authors.^{16,22} With this prophylactic procedure we decreased the risks of hemorrhagic complications, reducing blood flow from the descending cervical arteries and allowing the evacuation of the products of conception with a poor blood loss. In this series, the success rate for controlling the bleeding was 100%. This result is in agreement with a series of 8 cervical pregnancies treated by means of selective embolotherapy either as emergency or nonemergency procedure.²³ In this report, BUAE was performed with or without methotrexate administration, no case required the hysterectomy, and the control of the hemostasis was obtained in all cases.²³

The clinical course and follow-up was uneventful for our 2 patients who resumed normal menstruation 1 month after BUAE. Instead, the patient with the uterine myoma was treated with hysterectomy after 2 weeks because of a febrile

status resistant to antibiotic therapy because of ischemic degeneration of the myoma.

A comprehensive Medline search from 1975 revealed that this appeared to be the second case of cervical pregnancy with coexisting leiomyomata. In the first case reported,²⁴ the management of the cervical pregnancy consisted of BUAE followed by intraamniotic injection of methotrexate 70 mg. No remarkable bleeding was observed, and the patient only complained of amenorrhea until 11 months after the intervention.²⁴

Although a long-term outcome study on uterine embolization of leiomyomata reported the failure of the procedure, with failure defined as subsequent hysterectomy, myomectomy, or repeated embolization in 13.7%, 4.4%, and 1.6% of cases, respectively,²⁵ when the cervical pregnancy is associated with a uterine leiomyomata, the prognosis for future fertility could be poor, as noted in our third case. In this regard, with BUAE we intended to reduce blood loss and decrease the size of myoma. In fact, Has et al²⁴ showed a reduction in myoma size after BUAE of about 70%. Furthermore, myoma degeneration may be a potential com-

277 plication because of the deep and acute reduction in uterine
278 vascularization.

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Conclusion

283 Even if modern diagnostic and treatment options provide
284 an opportunity for conservative treatment of cervical preg-
285 nancy, during counseling it is pivotal to explain carefully
286 that potential complications can make radical treatment nec-
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PLACENTA, PRECLAMPSIA, ANEMIA

Intrauterine growth restriction (IUGR) is frequently associated with preeclampsia and is thus an important cause of maternal-fetal mortality and morbidity. (1) The disease is usually caused by uteroplacental vascular insufficiency (2) and results in chronic fetal hypoxia. As such, local tissue hypoxia in the placental bed has been proposed as the mechanism limiting trophoblast invasion into the spiral arteries.(3) Although maternal anemia may be linked to adverse pregnancy outcomes, the placenta is often enlarged, with increased angiogenesis in the fetal-placental vessels, to preserve gas transfer and fetal growth (4) while uterine artery blood flow often normal. (5) Some authors shown that interstitial invasion of extravillous trophoblast into uterine tissues is increased in anemia and reduced in preeclampsia.(6)

To further explore the relationship between tissue oxygenation and extravillous trophoblast invasion into the walls of uterine spiral arteries, the same authors studied intramural and endovascular trophoblast invasion in full-thickness placental bed biopsies at the time of cesarean hysterectomy in women whose pregnancies had been complicated by anemia or early-onset preeclampsia with IUGR or in normal pregnancies requiring intrapartum hysterectomy. Their data suggest that uteroplacental vascular insufficiency is due to excessive trophoblast apoptosis within the walls of spiral arteries rather than as a result of local hypoxic conditions inside the placental bed. They found that early-onset preeclampsia with IUGR is characterized by reduced invasion of the spiral arteries by extravillous (intramural) trophoblast. This disease is associated with a doubling in the

frequency of apoptosis and a halving in the mean lumen area of the spiral arteries. By contrast, maternal anemia displayed opposite findings, with increased numbers of infiltrating trophoblast cells, a similar frequency of apoptosis, and increased vasodilation of the spiral arterial lumens, compared with control subjects. In anemic women was observed an increased endovascular trophoblast invasion; during maternal anemia, increased invasion and further dilation of the lumens of spiral arteries by the invading trophoblast may be an adaptive response designed to increase oxygen transfer toward the placenta and fetus. (7) Previous studies have shown increased numbers of proliferating cells within placental villi and an alteration in the structure and density of peripheral gas-exchanging villi to favor gaseous exchange in anemia.(8)

Kadyroff et al revealed that adaptive processes in placentas of anemic women are not confined to placental villi but include maternal enhanced oxygen delivery at the level of the spiral arteries. With a deeper invasion⁸ and a further dilation of the uterine spiral arteries (in this study), more blood and thus more oxygen can be delivered toward the placenta and fetus. By contrast, there are opposite findings in the placental bed of women with early-onset preclampsia and IUGR, with fewer cells invading the walls of spiral arteries and an increased frequency of apoptosis of intramural trophoblast. Anemia is characterized by a normal partial pressures of oxygen of arterial blood but low oxygen content and is described as a clinical entity of systemic tissue hypooxygenation. (9) The increased invasion seen in anemia challenges the concept of trophoblast invasion being limited in preclampsia by local tissue hypoxia. Indeed, mild anemia may be a straightforward mechanism by which trophoblast invasion could be augmented in women at risk of this disease.

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To support and to confirm on clinical level the result of the study of Kadyrov we have evaluated the incidence of preclampsia in a group of women with thalassemic trait.

App. 18

Incidence of preclampsia in a group of pregnant women with thalassemic-trait

Introduction

Hypertension occurs in 5-8% of all pregnancies and may be complicated by preeclampsia. Preeclampsia is a complex clinical syndrome with insufficiently clear pathophysiology based on the damage of the vascular endothelium. As a result of this, generalized endothelial disruption in preeclampsia, a multiorgan dysfunction, can develop, most frequently reflected in the clinical presentation with haematological and renal damage and with a disordered function of the liver and central nervous system. Preeclampsia is a syndrome defined by hypertension (PA>140/90mmHg), that may be associated with proteinuria (>300mg/24h) and edema in pregnant women with previously normal blood pressure. This gestational complication occur in about 5-10% of pregnancies and with placental abruption, intrauterine growth restriction (IUGR) and intrauterine fetal death (IUFD) is the most common gestational vascular complication (*Younis 2003*). Preeclampsia is associated with maternal mortality and is the second cause of maternal death after thromboembolic complications. The etiology of preeclampsia is unknown, although much of the literature has focused on the degree of

trophoblastic invasion by the placenta, but also local environmental and iron deficiency are associated. In fact, during the hypoxic phase of placental development some transcription factors releasing calcitriol, erythropoietin, endothelial growth factor, **HIF 1 α** (hypoxic factor) were produced (*Safran 2003*). **HIF 1 α** is the promoter of production of **TGF β 3** that is the inhibitor of trophoblastic differentiation. This is an adaptive response to hypoxia that is increased in the early-onset preeclampsia (*Rajakumaz 2006*). However, preeclampsia is associated with increased production of **HIF 1 α** , **TGF β 3** and **ROS**, just as in altitude and in condition of hypoxia (*Solevmanlon 2005*). Some studies have disclosed that the iron supplementation in preeclamptic pregnancies increased the production of **ROS**, that are responsible of pathophysiology of preeclampsia (*Scholl 2005*). The hemoglobinopathies are a heterogeneous group of single-gene disorders that includes the structural hemoglobin variants and the thalassemias. More than 270 million people worldwide are heterozygous carriers of hereditary disorders of hemoglobin, and at least 300,000 affected homozygotes or compound heterozygotes are born each year (*Angastiniotis 1998*). The most common hemoglobinopathies are sickle cell disease and thalassemias. Sickle cell disease refers to a group of autosomal recessive disorders involving abnormal hemoglobin (hemoglobin S). The distorted red cells lead to increased viscosity, hemolysis, and anemia and a further decrease in oxygenation; it can cause repeated vasoocclusive crises with interruption of normal perfusion and function of several organs, including the spleen, lungs, kidneys, heart, and brain. Adults with Hb SS are functionally asplenic, having undergone autosplenectomy by adolescence. Absence of the spleen contributes to the increased incidence and severity of infection in patients with sickle cell disease. The most significant threat to patients with sickle cell disease is acute

chest syndrome that is characterized by a pulmonary infiltrate with fever that leads to hypoxemia and acidosis. The infiltrates are not infectious in origin but rather are due to vasoocclusion from sickling or embolization of marrow from long bones affected by sickling (*Duffy 2004*). The diagnosis of sickle cell disease is made by hemoglobin electrophoresis. In the homozygous form, nearly all the hemoglobin is Hb S with small amounts of Hb A₂ and Hb F. Heterozygous sickle cell trait (Hb AS) is identified by a larger percentage of Hb A and an asymptomatic course. Solubility tests (Sickledex) alone are inadequate for diagnosis because they cannot distinguish between the heterozygous AS and homozygous SS genotypes. Sickle cell disease is associated with increased pregnancy complications, and urinary tract infection (*American college of Obstetricians and Gynecologists 1993*). It may involve poor maternal nutrition, compromised uterine blood flow and placental infarction; endometritis is increased in women with sickle cell disease: cesarean section is the greatest risk factor for postpartum endometritis (*Monga 1993*). The thalassemias represent a wide spectrum of hematologic disorders that are characterized by a reduced synthesis of globin chains, resulting in microcytic anemia. Many different molecular mechanisms lead to thalassemia in populations from different areas of the world (*Kazazian 1990*). Alpha-thalassemia usually results from a gene deletion of two or more copies of the four α -globin genes. Deletion of one α -globin gene is clinically unrecognizable, and laboratory testing yields normal results. Deletion of two α -globin genes causes α -thalassemia trait, a mild asymptomatic microcytic anemia: individuals with these chromosomal abnormalities are referred to as carriers and are at an increased risk for having a child with a more severe form of thalassemia caused by deletion of three or four copies of the α -globin gene (α -thalassemia major).

Alpha-thalassemia trait is common among individuals of Southeast Asian, African, and West Indian descent. Alpha-thalassemia major (Hb Bart's) results in the absence of α -globin ($--/--$); this is associated with hydrops fetalis, intrauterine death, and preeclampsia (Davies 2000). Hemoglobin H disease usually is associated with mild to moderate

Number of Globin Genes	Genotype	Description	Clinical Features
4	$\alpha\alpha/\alpha\alpha$	Normal	Normal
3	$\alpha^-/\alpha\alpha$	Heterozygous $\alpha^{+/-}$ thalassemia Homozygous $\alpha^{+/-}$ thalassemia	Asymptomatic
2	α^-/α^- $\alpha\alpha/--$	Homozygous $\alpha^{+/-}$ thalassemia Heterozygous $\alpha^{\circ/-}$ thalassemia	Mild anemia
1	$\alpha^-/--$	$\alpha^{+/-}$ -thalassemia/ $\alpha^{\circ/-}$ thalassemia	Hb H disease Hemolytic anemia
0	$--/--$	Homozygous $\alpha^{\circ/-}$ thalassemia	Hb Bart's disease Hydrops fetalis

hemolytic anemia. Although alpha-thalassemia also may occur as a result of a gene mutation. In this case, the genes are present but the function is abnormal.

Beta-thalassemia is caused by a mutation in the β -globin gene that causes deficient or absent β -chain production, resulting in absence of Hb A. Individuals who are heterozygous for this mutation have β -thalassemia minor. Those who are homozygous have β -thalassemia major (Cooley's anemia) or a mild form called thalassemia intermedia. β -thalassemia major is characterized by severe anemia with resultant extramedullary erythropoiesis, delayed sexual development, and poor growth. Elevated levels of Hb F in individuals with β -thalassemia major partially compensate for the absence of Hb A; however, death usually occurs by age 10 years unless treatment is begun early with periodic blood transfusions. With transfusion, the severe anemia is reversed and extramedullary erythropoiesis is suppressed. β -thalassemia minor often occurs in association with Hb S (*Serjeant 2001*). β -thalassemia minor is common in individuals of Mediterranean, Asian, Middle Eastern, Hispanic, and West Indian descent, with various grade of severity, depending on the amount of β -chain production. The diagnosis of thalassemias is made by hemoglobin electrophoresis.

The World Health Organization has suggested a cut-off value of <6.8 mmol/l in the definition of anemia in pregnancy (*World Health Organization 1972*). In a population of unselected pregnant women there exists an inverse association between the women's hemoglobin concentration and the newborn's birth weight, which means that high hemoglobin levels are associated with low birth weight (*Murphy 1986*). High hemoglobin concentrations of >9 mmol/l increase blood viscosity, which may reduce placental perfusion and, thereby, the nutrition of the fetus, resulting in low birth weight (*Staer 1995*). In fact, preeclampsia and eclampsia are characterized by inadequate hemodilution, and these disorders are associated with low birth weight of the newborns; however,

anemic pregnancies decreased the rate of preeclampsia. Recently a study by Kadyrov compared pathological examination of 19 placentas: from 8 women with anemia, from 6 women with severe early-onset preeclampsia and from 5 controls, to compare trophoblast invasion into spiral arteries and relate these findings to trophoblast apoptosis. Trophoblast invasion into spiral arteries was severely impaired in preeclamptic pregnancies, with an increased trophoblast apoptosis and a reduced spiral artery lumen. On the contrary, trophoblast invasion into spiral arteries was increased in anemic pregnancies, with a similar trophoblast apoptosis and an increased spiral artery lumen. Infact, in preeclampsia the hypoxia is driving reduced invasion; by contrast, during maternal anemia, increased invasion and further dilation of the lumens of spiral arteries by the invading trophoblast may be an adaptive response designed to increase oxygen transfer toward the placenta and fetus (*Kadyrov 2005*).

Several studies demonstred that the rate of preeclampsia is decreased in anemic pregnancies because trophoblast invasion into spiral arteries is increased in anemic pregnancies and severely impaired in preeclamptic pregnancies (*Kadyrov 2003*). Our purpose is to determine the incidence of preeclampsia in pregnancies complicated by hemoglobinopathies to evaluate if hemoglobinopathies is a protective factor preventing the development of preclampsia.

MATERIALS AND METHODS

In the period between 1993 and 2006, 290 patients with thalassemia trait were followed at our Ambulatory of High Risk Pregnancy. Subjects were screened at the prenatal diagnosis for thalassemia using chorionic villi obtained by chorionic villus sampling (CVS) at 9-13 weeks of gestation. All patient with thalassemia trait were enrolled in our

study to evaluate maternal and neonatal outcome; the follow-up was centred about the incidence of preeclampsia, with screening for blood pressure, proteinuria, edema, abital and actual weight. Were excluded from follw-up patients with fetus affected at prenatal diagnosis for failure of pregnancy. Moreover all patient who miscarried, in spite of the fetus was healty at prenatal diagnosis, were excluded. To test this hypothesis, we studied a cohort of 219 pregnant women and 553 low-risk women who did not take prenatal invasive diagnosis, but were enrolled at the time of prenatal ultrasonographic screening of nuchal translucency. The diagnosis of preeclampsia was determined by increased blood pressure accompanied by proteinuria. Preclampsia was defined as blood pressure > 140/90 mmHg on two or more occasions, occurring after 20 weeks of gestational age. . Proteinuria is defined as the urinary escretion of 0,3 g protein or greater in a 24 hour specimen.

RESULTS

290 CVS for thalassemia, 50 fetus (17,2%) resulted positive at the molecular diagnosis of Thalassemia and pregnancy failed. Of the 240 women with thalassemia trait, 8 patients (3,3%) were excluded for miscarriage, 13 women (5,4%) lost during follow-up.

Therefore, a total of 219 patients (75,5% of 290 women) with thalassemia trait but fetus healty at prenatal diagnosis were included in our study. Our purpose was to determine the rate of preeclampsia for evaluate the association between preeclampsia and thalassemia.

Forthyeight (8.6%) women in the control group developed PIH compared with five (2,2%) in the case group. The odds of developing the disease were significantly lower

in the treated subjects ($P < 0.001$). The patients with preeclampsia delivered with cesarean section and gestational age at delivery was between 36 and 41 weeks. Birth weight was between 2880 ± 870 g. Of the 219 patients, 6 (2,7%) had alone edema without hypertension or proteinuria: 5 women (83,3%) are delivered with cesarean section between 38 and 40 weeks of gestation, alone a patient (16,6%) with vaginal delivery at 38 week. Of the 219 patients, 6 (2,7%) had proteinuria and hypertension without edema or edema with hypertension without proteinuria: three cases of proteinuria with hypertension (50%) and delivery through cesarean section between 38 and 40 weeks and three cases of edema with hypertension (50%) ; of these three cases, two women (66,6%) are delivered with cesarean section between 35 and 37 weeks and alone a women (33,3%) with vaginal delivery at 39 week of gestation. Birth weight was between 1750g and 3750g. Of the 6 patient, alone a women(16,6%) with edema and hypertension had antihypertensive therapy: she is delivered at 35 week of gestation with cesarean section and birth weight was 1750g. Finally, of the 219 patients, 3 (1,3%) had gestational hypertension, that occurs after 20 week of gestation in a woman previously normal blood pressure. 2 of 3 patients (66,6%) had antihypertensive therapy until delivery; on the contrary, a patient (33,3%) not had therapy. All three patients are delivered through cesarean section between 37 and 40 weeks and birth weight was between 2750g and 4100g.

DISCUSSION

Our study confirm on clinical level the data of Kadyrov showing a protective factor deriving from anemic state in pregnancy with decreases of the incidence of preeclampsia and of the other gestational vascular complications. Infact, the rate of preeclampsia in women with thalassemia trait was four fold low. There is an inverse relationship between preeclampsia and anemia because trophoblast invasion into spiral arteries was severely impaired in preeclamptic pregnancies, that the hypoxia is driving reduced invasion; on the contrary, trophoblast invasion into spiral arteries was increased in anemic pregnancies as an adaptive response designed to increase oxygen transfer toward the placenta and fetus (*Kadyrov 2005*). On the other hand, is very important a management of these disorders for maternal and neonatal outcome.

Preeclampsia predispose to potentially lethal complications such as abruptio placentae, acute renal failure, cerebral hemorrhage, disseminated intravascular coagulation (CID) and circulatory collapse (*Chesley 1984*). They also weigh heavily in fetal and neonatal morbidity and mortality. Much of the neonatal mortality is attributable to premature delivery, miscarriage and intrauterine growth restriction (IUGR) that is necessary for the obstetrician to provide meticulous surveillance of fetal growth and well-being, including the biophysical profile, nonstress test, amniotic fluid volume measurement, and Doppler velocimetry of fetal vessels (*Resnik 2002*). Although absence or reversal of end-diastolic flow in the umbilical artery is suggestive of poor fetal condition, normal umbilical Doppler flow is rarely associated with significant morbidity and likely represents a constitutionally small fetus (*Ott 2000*). Therapy of preeclampsia consists in fetal and maternal evaluation: fetal evaluation consists of nonstress tests, biophysical profiles,

daily fetal movement assesment, ultrasound examination with Doppler velocimetry to evaluate amniotic fluide and intrauterine growht restriction; hospitalization is often initially recommended for women with new-onset preeclampsia (*Report of the National High Blood Pressure Education 2000*). Maternal evaluation consists primarily of frequent evaluation for worsening preeclampsia. Initial laboratory tests consist of evaluation of platelet count, liver enzymes, and renal function and a 12-hour to 24-hour urine collection for protein; infact, the two main goals of management of women with preeclampsia are prevention of seizures or eclampsia and control of hypertension. The data support the use of magnesium sulfate to prevent seizures in women with severe preeclampsia or eclampsia (*Witlin 1998*), during that fetal bradycardia frequently occurs. Antihypertensive therapy is generally recommended for women wiht preeclampsia and alfametildopa and nifedipin are the two agents most commonly used for this purpose; diuretics and inhibitors-ACE are strictly not recommended (*Cunningham 2001*).

The decision to deliver a patient with preeclamspia must balance both the maternal and fetal risks and delivery represents the final therapy of this disorder. If maternal and fetal conditions are stables the pregnancy can continue to term. If maternal conditions fall off but fetal conditions are stables, since 34 week of gestation women can deliver after therapy with corticosteroids to induce pulmonary maturity. Induction of labor with cesarean delivery in women with severe preeclampsia remote from term is convenient if fetal conditions fall off (*Nassar 1998*). Much of the obstetric research in the past several decades has been directed at findings ways to prevent preeclampsia and eclampsia. Recent studies have focused on low-dose aspirin, antioxidant therapy and calcium supplementation. Most evidence suggests that low-dose aspirin therapy is of little, if any,

benefit in preventing preeclampsia in low-risk women: 60-150 mg/daily (*Heyborne 2000*). Recently, antioxidant therapy with 1,000 mg per day of vitamin C and 400 mg per day of vitamin E has shown promise in preventing preeclampsia (*Chappell 1999*). While preeclampsia is a gestational vascular complication that gets well with delivery, hemoglobinopathies are disorders of hemoglobin that not get well with delivery. If both parents are determined to be carriers, genetic counseling is recommended. A complete blood count (CBC) is the appropriate initial laboratory test with determination of mean corpuscular volume (MCV): in fact, patients who have a low MCV (less than 80 fl) may have one of the thalassemia traits and are candidates for hemoglobin electrophoresis. Beta-thalassemia is associated with elevated Hb F and elevated Hb A₂ levels (more than 3.5%). Neither hemoglobin electrophoresis nor solubility testing can identify individuals with alpha-thalassemia trait; only molecular genetic testing can identify this condition. Prenatal diagnostic testing for alpha and beta-thalassemia is possible if the mutations and deletions have been previously identified in both parents. These DNA-based tests can be performed using chorionic villi obtained by chorionic villus sampling (CVS) at 10-12 weeks of gestation to early characterize fetus affected by disorder of hemoglobin.

The course of pregnancy in women with the alpha-thalassemia trait is not significantly different from that of women with normal hemoglobin. Pregnancy in women with beta-thalassemia major is extremely rare; initially this was because delay of growth and sexual development and early death in untreated patients prevented reproduction. After the introduction of transfusion therapy in the 1960s, pregnancy was still uncommon because of infertility (secondary to hypothalamic dysfunction and anovulation caused by hemosiderin deposition) (*Jensen 1995*). Since the introduction of hypertransfusion and

iron chelation therapy with deferoxamine, several reports have documented favorable pregnancy outcomes. However, pregnancy is recommended only for those with normal cardiac function who have had prolonged hypertransfusion therapy to maintain hemoglobin levels at 10 g/dL and iron chelation therapy with deferoxamine (*Aessopos 1999*). Fetal growth should be monitored with serial ultrasonography and the mode of delivery should be individualized. Beta-thalassemia minor usually causes mild asymptomatic anemia and pregnancy outcome is favorable. No differences are noted in perinatal outcomes such as low Apgar scores, congenital malformations, or perinatal mortality (*Sheiner 2004*). Pregnancy in women with sickle cell disease is associated with an increased risk of morbidity and mortality because the combination of underlying hemolytic anemia and multiorgan dysfunction associated with this disorder: patients have increased risk for preterm labor, premature rupture of membranes, antepartum hospitalization and postpartum infection (*Sun 2001*). The most common cause of recurrent morbidity in Hb SS disease is painful crises and hydroxyurea has been shown to reduce the frequency of painful crises but is not recommended during pregnancy because it is teratogenic. Opiates and oxygen are can replace hydroxyurea, on the other hand is necessary a multidisciplinary approach to evaluate painful crises (*Rees 2003*). Pregnant patients with sickle cell disease need increased prenatal folic acid supplementation: 4mg per day. There is no consensus regarding the use of transfusion to lower the percentage of Hb S to approximately 40% while simultaneously raising the total hemoglobin concentration to about 10 g/dL. Prophylactic transfusion is associated with a decreased risk for painful crises and severe anemia, but no differences are observed for pregnancy

outcome (*Koshy 1988*). Such as thalassemias and preeclampsia, the mode of delivery should be individualized.

Our results suggest that anemic state of pregnant women is a protective factor versus the onset of preeclampsia and in the future a possible treatment possible with iron chelant or absence or minor administration of iron during pregnancy can reduce the incidence of PIH, thus opening new perspectives in its prevention and therapy.

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