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DOTTORATO DI RICERCA IN
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Dipartimento di Scienze Cliniche

NEW SCREENING TESTS IN EARLY PREGNANCY: DIAGNOSIS AND PREVENTION

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INDEX

PRESENTATION

"New screening tests in early pregnancy: diagnosis and prevention" is the title chosen to present two papers I published during these three years.

Conserva V, Signaroldi M, Mastroianni C, Stampalija T, Ghisoni L, Ferrazzi E. *Distinction between fetal growth restriction and small for gestational age newborn weight enhances the prognostic value of low PAPP-A in the first trimester.* Prenat Diagn. 2010 Oct;30(10):1007-9

Conserva V, Muggiasca M, Arrigoni L, Mantegazza V, Edoardo Rossi E, Ferrazzi E. *Recurrence and severity of abnormal pregnancy outcome in patients treated by low-molecular-weight-heparin: a prospective pilot study*. J Matern Fetal Neonatal Med. 2011 Nov 29

These two paper deal with subjects that are apparently different: the first paper studies the prognostic value of a placental marker measured in the first trimester, PAPP-A, for abnormal pregnancy outcomes, the second one investigates the possible role of low molecular weight heparin to improve the development of pregnancy in patients with previous abnormal pregnancy outcomes. Besides the specific clinical application of both results, the two studies assume the central role of placentation. A physiologic communication and balance in maternal-fetal interface allows a correct development of placenta from the earliest days of gestation to twenty four weeks of pregnancy, although the phenotypic outcome of this process can be clinically evaluated much later in pregnancy. These two papers tap into this silent and fundamental process in order to evaluate a potential for early diagnosis or prevention.

ABSTRACTS

Distinction between fetal growth restriction and small for gestational age newborn weight enhances the prognostic value of low PAPP-A in the first trimester.

Conserva V, Signaroldi M, Mastroianni C, Stampalija T, Ghisoni L, Ferrazzi E.

Prenat Diagn. 2010 Oct;30(10):1007-9

Objective Low levels of PAPP-A in maternal blood may become an early marker of obstetrical complications. The aim of this article was to sort out those outcomes consistently related to an abnormal placental vascular function and to evaluate their association with low levels of maternal serum PAPP-A in early pregnancy

Methods We analyzed retrospectively a database of the first trimester combined screening of an Italian biotech company and investigated the correlation between PAPP-A value < 5th percentile, 0.40 multiples of the median (MoM), and infants with birthweight below the 10^{th} percentile for gestational age (small for gestational age SGA), preterm delivery, GH and PE not associated with intra-uterine growth restriction (IUGR), IUGR isolated with an abnormal umbilical PI or associated with maternal GH-PE, placental abruption and intra-uterine fetal demise (IUFD) after 22 weeks of gestation.

Results 1687 patients were analyzed. Overall pregnancy complications were observed in 31.4% of women with low PAPP-A and in 21.1% women with a PAPP-A value >0.4 MoM ($P < 0.0001$).
Severe and early fetal growth restriction (<34 weeks) with abnormal umbilical PI or maternal PE, was significantly associated with low levels of PAPP-A (OR= 10, 95% CI 1.0–97, $P = 0.02$). No relationship was observed between SGA newborns and low PAPP-A. Weak association was observed in with GH and PE not associated with fetal growth restriction (OR = 1.9, 95% CI 1.1–3, $P = 0.01$). We also observed a correlation between low PAPP-A and preterm delivery (OR = 1.8, 95% CI 1.1–2,9, $P = 0.01$). Because of the small number of cases, the OR for placental abruption and IUFD were not calculated

Conclusions. Low values of PAPP-A are associated with abnormal obstetrical outcome. Evaluating separately growth-restricted fetuses and small for gestational age fetuses we observe that only growth-restricted fetuses are significantly associated with low values of PAPP-A, whereas SGA newborns, simply defined by their percentile rank, are not predicted by this test in the first trimester of pregnancy.

Recurrence and severity of abnormal pregnancy outcome in patients treated by low-molecular-weight-heparin: a prospective pilot study.

Conserva V, Muggiasca M, Arrigoni L, Mantegazza V, Edoardo Rossi E, Ferrazzi E

J Matern Fetal Neonatal Med. 2011 Nov 29

Objective This prospective pilot study assesses the recurrence rate and severity of abnormal pregnancy outcome (APO), excluding early pregnancy complications, in pregnant patients, without acquired thrombophilia, treated by prophylactic doses of LMWH, independently from their congenital thrombophilic condition.

Methods We recruited a cohort of 128 pregnant patients with previous APO; 100 of whom with APO and intrauterine growth restriction (IUGR), and 28 with maternal APO only. LMWH treatment was started at recruitment. Composite cross over recurrence rate IUGR, gestational hypertension, preeclampsia, HELLP, abruptio was analyzed. The main outcome measure was severe APOs with iatrogenic delivery ≤ 32 weeks of gestation.

Results Median gestational age at LMWH treatment was 20 weeks. Severe APO decreased in treated pregnancies from 45% to 4% (R.R.=0.3, CI .95=0.2-0.8). This value was not significantly different in thrombophilic and non thrombophilic patients. When severe and minor complications were analyzed altogether the recurrence rate was 28%. In patients with APO and FGR in the index pregnancy, newborn weights were significantly better in the treated pregnancy: 1090g (1035-1145) vs. 850g (535-1200), P<0,01)

Conclusions. Prophylactic regimen of LMWH significantly reduced the recurrence rate of severe composite APO in pregnancies affected in the index pregnancy by APO and fetal growth restriction or SGA newborns. This result was independent from the patients' inherited thrombophilic conditions.

GENERAL INTRODUCTION

Prior to implantation, the outer wall of the blastocyst is formed by a single layer of uninucleate trophectoderm cells. Contact with the maternal uterine interface induces local differentiation of this layer into an outer multinucleated syncytiotrophoblast, and an underlying population of progenitor cells, the cytotrophoblast cells (1).

The maternal-fetal interface has certain unique features immunologically. It must exhibit immune tolerance to the allogenic syncytiotrophoblast, cytotrophoblast and fetus, and at the same time, it must maintain adequate protection against microbial invasion of the maternal host. The syncytiotrophoblast forms the terminally differentiated epithelial covering of the placental villous trees, being in direct contact with maternal blood and behaves, together with decidual cells, as "non-classical" immune cells at the utero-placental interface expressing toll-like receptors (TLR). These receptors at maternal-fetal interface not only recognize microorganisms, but also encode for genes resulting in release of cytokines, prostaglandins and coagulation factors (2).

Cytotrophoblast cells can differentiate along either of two pathways. They may fuse with the overlying syncytiotrophoblast, thus increasing its mass. Alternatively, where villi make contact with the decidua they may differentiate along the extravillous pathway and invade the maternal tissues. In doing so, they start as interstitial trophoblast invading through the stroma of the decidua and then may remain interstitial or may follow two other routes, towards the lumen of spiral arteries as endovascular trophoblast (3), or towards the lumen of uterine glands as endoglandular trophoblast (4). The interstitial trophoblast normally penetrates as far as the inner third of the myometrium.

Trophoblast development and differentiation are crucial processes for a physiologic pregnancy development. Any insult resulting in an aberrant development and differentiation of the villous syncytiotrophoblast poses a risk for the integrity of the placental barrier and the release of necrotic and aponecrotic trophoblast fragments, culminating in a systemic inflammatory response of the mother (5). This process is shown by the shift of normal Th1/ Th2 balance to Th2/ Th1, resulting in increased production of inflammatory cytokines (6). Inflammation is known to play a pivotal role in pathogenesis of Preeclampsia (PE), and other obstetric complications including pretermlabor and chorioamnionitis (7) by the activation of circulating leukocytes in maternal blood, along with increased levels of particular subsets of leukocyte-derived microparticles (MP). Other markers of inflammatory change include Interleukin-1 Receptor Antagonist (IL-1RA) and tumor necrosis factor (TNF)-R1in women with PE, as compared to non-pregnant cohort (8). Local hemostatic changes in placental vasculature have been well defined (9). While there is expression of tissue factor (TF) in the synctiotrophoblast, there is a concomitant decrease in the concentration of tissue factor inhibitor pathway (TFPI) in the synctiotrophoblast. Furthermore, the balance between TF-

TFPI has been found to be altered in women with gestational vascular complications. Select coagulation factors and global markers of coagulation activation have been partially characterized in women with placenta mediated complications. For example in women with pre eclampsia, coagulation abnormalities which have been identified include decreased antithrombin activity, elevated PAI-1 antigen and elevated Thrombin-Antithrombin (TAT) complexes (10).

Biochemical and immunologic markers reflect the systemic responses of maternal systems to abnormal pregnancy and may be of an inflammatory or metabolic origin.

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PART I

DISTINCTION BETWEEN FETAL GROWTH RESTRICION AND SMALL FOR GESTATIONAL AGE NEWBORN WEIGHT ENHANCES THE PROGNOSTIC VALUE OF LOW PAPP-A IN THE FIRST TRIMESTER

INTRODUCTION

The placentation, placenta itself and its myriad functions are central to successful reproductive outcomes. The placenta is the first complex mammalian organ to develop and as development continues, it becomes a robust endocrine organ, producing a spectrum of hormones that are unique to the pregnancy, such as human chorionic gonadotropin, estriol, as well as hormones identical to those produced elsewhere, such as estrogen and progesterone. This hormone production is intrinsically coupled to the physiologic changes of pregnancy and is critical for pregnancy maintenance.

Biochemistry and biosynthesis of PAPP-A

Pregnancy-associated plasma protein-A (PAPP-A) was first identified in 1974 (1). Circulating PAPP-A is principally derived from the syncytiotrophoblast (2,3), and the PAPP-A gene is located on human chromosome 9q33.1 (4). The protein is secreted as a disulfide-bound homodimer with a molecular weight of 400,000 g/mol (5). Each subunit consists of 1,547 amino acids (6) and includes modules known to occur in other proteins (Figure 1), but the degree of identity is generally low. A distinguishing feature is the metalloproteinase modules (MP), based on which PAPP-A is classified as a metzincin metalloproteinase, and hence related to the large family of matrix metalloproteinases. Other modules of PAPP-A include three Lin12-Notch repeat (LNR1-3) modules, a single laminin G-like module (LG), and five complement control protein modules (CCP1-5) that mediate binding of PAPP-A to heparan sulfates of cell surfaces.

Almost all of the circulating PAPP-A is covalently bound to proMBP (proform of eosinophil major basic protein). During pregnancy, both PAPP-A and proMBP are expressed abundantly in the human placenta (7), but in different cell types. PAPP-A is principally expressed in the syncytiotrophoblast, whereas proMBP is expressed in extravillous cytotrophoblasts (2), from where it is secreted without propeptide cleavage. Thus, the process of PAPP-A/proMBP complex formation occurs in the extracellular environment, probably at the surface of the syncytiotrophoblast. PAPP-A bound to the cell surface readily forms a complex with proMBP, this complex is unable to bind to the cell surface and therefore enters the circulation.

PAPP-A and the insulin-like growth factor system

Strong evidence suggests that PAPP-A increases the local bioavailability of insulin-like growth factor (IGF) by cleaving the inhibitors IGFBP-4 and −5 (insulin-like growth factor-binding protein-4 and −5) (8). IGF is considered mitogenic and anti-apoptotic and is important for the growth of human cells in most tissues (9). This probably explains why PAPP-A knockout mice are reduced to 60% of normal size at birth, strikingly similar to the IGF-II knockout mouse (10). Only dimeric PAPP-A, not the PAPP-A/proMBP complex, shows this proteolytic activity (5). At term, about 1% of the circulating PAPP-A is present as uncomplexed, active PAPP-A. However, the fraction of active PAPP-A is much higher in the first trimester, although the total level of PAPP-A is lower.

Maternal serum levels of PAPP-A

Maternal PAPP-A blood levels become detectable soon after implantation and increase throughout pregnancy, with a doubling time of 3–4 days during the first trimester and maximum levels at term (11,12) The rapid increase in PAPP levels during the first trimester causes the interpretation of a given value to be very dependent on gestational age. Common practice is therefore to use the unit MoM (Multiples of Median) as a gestational age-independent expression of PAPP-A concentration. The average half life of PAPP-A after normal delivery is 53 ± 26 hours (mean plus SD) (13). In addition to gestational age, a number of maternal and pregnancy-associated characteristics affect the maternal serum concentration of PAPP-A (*Table 1*). Some of these (multiple parity and smoking) are probably related to the mass of trophoblastic tissues, because the PAPP-A concentration increases with increasing ultrasound-determined placental volume (14). Other factors such as maternal pre-pregnancy weight have been correlated with the volume of distribution, whereas associations with parity, ethnicity, and assisted reproduction have so far escaped biological explanations. Several studies have tested the hypothesis that low maternal serum levels of pregnancy-associated plasma protein A (PAPP-A) may predict adverse pregnancy outcomes other than Down syndrome in first trimester (15- 20). Low levels of PAPP-A in maternal blood could become an early marker of obstetrical complications associated with poor placental function, that is small babies, gestational hypertension (GH), pre-eclampsia (PE), stillbirth and even premature delivery. However, contradictory results had been observed in different cohorts (19-21). These findings could result as a consequence of non-homogeneous criteria in the definition of different abnormal obstetrical outcomes. Recently, Poon et al. (22) showed that low PAPP-A is significantly associated with early PE but not with late PE. This data confirmed the need to distinguish among abnormal obstetrical outcomes those based on similar placental damage. Moreover, when considering poor obstetrical outcomes, other environmental factors have to be taken into account.

Among these, maternal smoking has been largely studied while it is associated with impaired fetal growth. Therefore, this factor could interfere when evaluating the predictive value of PAPP-A.

AIM OF THE STUDY

The aim of this article was to sort out among abnormal obstetrical outcomes those consistently related to an abnormal placental vascular function and to evaluate their association with low levels of maternal serum PAPP-A in early pregnancy.

METHODS AND RESULTS

The database of the first trimester combined screening of an Italian biotech company (Bi-Tech Ltd.), certified by the Fetal Medicine Foundation (FMF), was analysed retrospectively by obstetrics and gynaecology specialists at the University Department of Clinical Sciences, University of Milan, under the regulations of a research contract. The database was searched for all combined first trimester screening tests in women with singleton pregnancies performed between January 2007 and January 2008. All women with high risk for trisomy 21, defined as risk greater than 1/350, were identified together with the women who presented intermediate risk, defined as risk 1/350 ÷ 1/1000. A control group of pregnant women at low risk for Down syndrome (<1/1000) was selected by means of a randomization table. The risk was evaluated by the combination of maternal age, nuchal translucency (NT) measurement, maternal serum-free β-hCG and PAPP-A. NT had been measured according to the FMF guidelines (23), while Bi-Tech Ltd. performed the analysis of serum metabolites and calculated the combined risk. PAPP-A values were corrected for maternal weight and smoking habit. Pregnancy outcome of all patients was obtained by telephone interviews conducted by a physician. Women with an aneuploid fetus diagnosed by invasive procedures or at birth filled an outcome form provided by the Bi-Tech company. In our cohort, the PAPP-A 5th percentile corresponded to 0.40 multiples of the median (MoM). This was used to define a low PAPP-A value and it is in accordance with previously observed results by Krantz *et al*. (16) and Dugoff *et al*. (16), respectively, 0.42 and 0.45 MoM. The following pregnancy complications were evaluated: (1) infants with birthweight below the 10^{th} percentile for gestational age (small for gestational age SGA); (2) preterm delivery <37 weeks of gestation; (3) GH and PE not associated with intra-uterine growth restriction (IUGR), the definition of GH and PE was according to the criteria proposed by Davey and MacGillivray (1988); (4) IUGR isolated or associated with GH or PE. IUGR was defined as decrement of the fetal abdominal circumference below the 10th percentile for local standard intra-uterine growth curves associated with an abnormal umbilical PI or maternal GH-PE. We also analysed the correlation of low PAPP-A with (5) placental abruption and (6) intra-uterine fetal demise (IUFD) defined as fetal death after 22 completed weeks of gestation. Patients with more than one complication were counted only once, taking into account the most severe complication as shown in increasing order in this list. We performed univariate logistic regression model with maximum likelihood fitting to evaluate the correlation between PAPP-A and poor obstetrical outcomes and to estimate odds ratios (OR) and their corresponding 95% confidence interval (CI). To account simultaneously for the effects of maternal smoking and weight, we also performed a multiple logistic regression model (25). Statistical significance was assumed for $P < 0.05$. Over the 12-months period, we identified 401 women with high risk and 867 with

intermediate risk for Down syndrome, while 502 subjects were randomly selected out of a larger sample (13 376) of women at low risk for chromosomal aneuploidies. The final sample size counted 1770 participants. Pregnancy outcomes were available for the whole study population. Chromosomal abnormalities were diagnosed in 26 subjects and were excluded from the analysis. Spontaneous miscarriage <18 weeks of gestation occurred in 7 women. Gestational diabetes developed in 50 patients. These patients were also excluded from the final analysis, and the remaining 1687 patients were considered for the purpose of this study. Overall, pregnancy complications were observed in 31.4% (132/421) of women with low PAPP-A and in 21.1% (267/1266) women with a PAPP-A value >0.4 MoM ($P < 0.0001$). No significant differences between low and normal PAPP-A groups were observed for ethnicity (99.1% Caucasians vs 99.5%) and maternal age (33 years, i.r. 31–35 vs 33 years, i.r. 31–36). The frequency of smoking habit (9.5 vs 6.2%, P value = 0.03) and maternal weight (63 kg, i.r. 56–72 vs 60 kg, i.r. 55–67, P value <0.001) were significantly different in two groups. Table 2 shows the comparison for the considered outcomes in the two groups, pregnant patients with low and normal PAPP-A and not adjusted and adjusted OR. Because of the small number of cases, the OR for placental abruption and IUFD were not calculated. Similarly, the distinction between the early (<34 weeks) and late onset (≥34 weeks) of adverse outcome was possible just for the IUGR group. The highest OR for abnormal outcome was observed for IUGR below 34 weeks of gestation, followed by adjusted OR for preterm delivery. SGA newborns were observed in about 10% of cases, both in women with low and normal PAPP-A.

DISCUSSION

We observed that PAPP-A values below 0.40 MoM (5th centile) in first trimester confer a higher relative risk for complications caused by placental vascular insufficiency and preterm delivery. Severe fetal growth restriction, in association with vascular placental damage as assessed by an abnormal umbilical PI or maternal PE, was significantly associated with low levels of PAPP-A in early pregnancy. When this association was evaluated for severe and early onset IUGR (<34 weeks), the OR predicted by low PAPP-A in this cohort was up to 10 (95% CI 1.0–97, P = 0.02). By contrast, no relationship was observed between SGA newborns and low PAPP-A in the first trimester, whereas most studies demonstrated a positive predictive value of low PAPP-A for small for gestational age fetuses. Our result could be explained by the fact that we separated from the SGA group the IUGR group, that is those newborns whose growth was restricted due to severe placental insufficiency, as defined by an abnormal umbilical PI or maternal GH/PE. According to these criteria, the incidence of SGA that we observed in this cohort was around 10% as expected when the 10th percentile of a reference population is adopted to sort out SGA newborns. SGA are considered, by the definition itself, a normal biological variant, not necessarily associated with growth restriction. Weak associations were observed in pregnant women with GH and PE not associated with fetal growth restriction (not adjusted OR = 1.9); moreover, the OR adjusted for smoking habits and maternal weight was not significant. Thus, this ratio may not be clinically meaningful. We also observed a correlation between low PAPPA and preterm delivery (adjusted and not OR = 1.8). Preterm delivery is mainly caused by infection, placental vascular damage and stress (26). Therefore, it is conceivable from a biological point of view that low levels of PAPP-A in early gestation may result from vascular damage. We acknowledge that the main criticism to our report could be the small number of cases in the cohort. This is the reason we were not able to evaluate the correlation between the low PAPP-A and intra-uterine fetal death and placental abruption, as well as the distinction between early and late onset of adverse outcomes for all groups As reported (15,16,18), many of the associations between low PAPP-A levels and the adverse outcomes are statistically highly significant, but the sensitivity and positive predictive values for the individual outcomes are relatively low: one in ten pregnancies with low PAPP-A concentration resulted in PE with severe IUGR or severe IUGR with an abnormal umbilical PI. We conclude that low values of PAPP-A confirm their association with abnormal obstetrical outcome. However, the distinction between growth-restricted fetuses and small for gestational age fetuses allowed us to prove that only growth-restricted fetuses are significantly associated with low values of PAPP-A, whereas SGA newborns, simply defined by their percentile rank, are not predicted by this test in the first trimester of pregnancy.

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Figure 1 legend

The protein is secreted as a disulfide-bound homodimer with a molecular weight of 400,000 g/mol. Each subunit consists of 1,547 amino acids and includes modules known to occur in other proteins, but the degree of identity is generally low (A). Other modules of PAPP-A include three Lin12-Notch repeat (LNR1-3) modules, a single laminin G-like module (LG), and five complement control protein modules (CCP1-5) that mediate binding of PAPP-A to heparan sulfates of cell surfaces.

Figure 1.

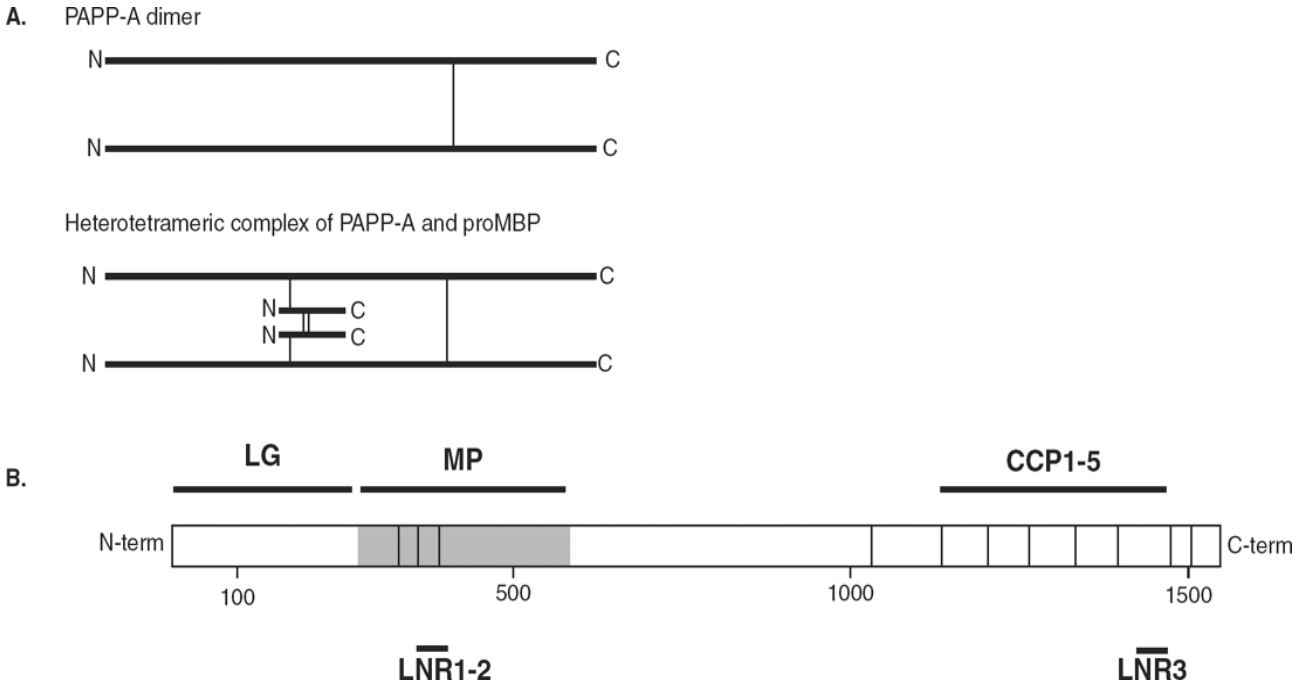
A.
PAPP-A dimer
N
C
N
C
Heterotetrameric complex of PAPP-A and proMBP
N
C
N
C
N
C
N
C
B.
LG
MP
CCP1-5
N-term
C-term
100
500
1000
1500
LNR1-2
LNR3

Table 1. Maternal and pregnancy-related factors influencing the level of PAPP-A in maternal serum during pregnancy.

Twins	1.86–2.12 MoM
Dichorionic	2.25 MoM
Monochorionic	1.76 MoM
Mode of conception	
IVF	0.80–0.90 MoM
ICSI	0.66–0.81 MoM
Ethnicity (ratio)	
Afro-Carribean vs. Caucasian	1.55–1.57 MoM
South Asian vs. Caucasian	1.03–1.08 MoM
East Asian vs. Caucasian	1.09–1.20 MoM
Maternal pre-pregnancy weight	
35–45 kg vs. all	1.55 MoM
115–125 kg vs. all	0.42 MoM
Maternal smoking during pregnancy	
Smoking in pregnancy	0.82–0.86 MoM
Nulliparous vs. multiparous	1.01–1.02 MoM
IDDM vs. no IDDM	0.80–1.01 MoM
Maternal HIV-infection vs. HIV-negative	0.84 MoM

ICSI, intracytoplasmic sperm injection; IDDM, insulin dependent diabetes mellitus; IVF, in vitro fertilization; MoM, multiples of the median; PAPP-A, pregnancy-associated plasma protein-A.

Table 2 —Abnormal outcome in pregnant women with PAPP-A ≤0.40 MoM and >0.40 MoM and not adjusted and adjusted OR for maternal smoking and weight

	PAPP-A ≤0.40 MoM (*n* = 421) *n* (%)	PAPP-A >0.40 MoM (*n = 1266*) *n* (%)	Not adjusted OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
Normal outcome	289 (69)	999 (79)				
SGA	48 (11.4)	131 (10.3)	1.1 (0.8–1.0)	0.54	1.3 (0.9–1.9)	0.10
IUGR+ PE/GH or IUGR	27 (6.4)	36 (2.8)	2.3 (1.4–3.9)	0.001	2.7 (1.6–4.6)	0.0002
<34 weeks	*5*	*1*	*10 (1.0–97)*	*0*.02		
≥34 weeks	*22*	*35*	*1.9 (1.2–3.4)*	*0*.01		
GH-PE*	27 (6.4)	45 (3.6)	1.9 (1.1–3.0)	0.01	1.5 (0.9–2.4)	0.14
PTD	27 (6.4)	46 (3.6)	1.8 (1.1–2.9)	0.01	1.8 (1.1–2.9)	0.02
Abruptio	2 (0.5)	4 (0.3)				
IUFD >22 *weeks*	1 (0.2)	5 (0.4)				

* Twenty five patients with PE; 4 <34 weeks of gestation.

PART II

RECURRENCE AND SEVERITY OF ABNORMAL PREGNANCY OUTCOME IN PATIENTS TREATED BY LOW-MOLECULAR-WEIGHT –HEPARIN: A PROSPECTIVE PILOT STUDY

INTRODUCTION

A dynamic balance of inflammatory cellular and molecular mechanisms dominates placental development and function to its end. An unbalanced inflammatory activation seems to play a dominant role for placental lesions linked to abnormal pregnancy outcomes (APO) such as IUGR, preeclampsia and abruption [1-2].

Accordingly, we hypothesize that low molecular weight heparin (LMWH) may be useful in patients with APO; mainly because of the role LMWH may play in the regulation of inflammation [3-4-5] rather than on its antithrombotic role [6-7].

Potential benefits of LMWH could be due to enhancement of metalloproteasis, which may cause an improvement of trophoblastic invasion [8], or by its antiapoptotic role in placental cells [9], or even by its key role in complement activation inhibition 11 [10].

This background, together with many other recent studies induces to reconsider the original hypothesis of an antithrombotic usage of LMWH to prevent and treat abnormal obstetrical outcome. Indeed, many studies, challenging the correlation between congenital thrombophilia with APO, came almost to a dead end [11-12]. None of the large studies designed to achieve an adequate power observed but a weak odd ratio between congenital thrombophilia and APO [7].

Actually, Rey and co-workers [13] completely readdressed this issue of LMWH and thrombophilia simply by including in a randomized trial of LMWH only pregnant patients with previous APO, without thrombophilic conditions. In this case, patients treated with LMWH showed a significant reduction of APO versus non-treated patients. More recently Gris found similar significant reduction of recurrence of ischemic placental disease in pregnant women with previous HELLP syndrome randomized to LMWH vs. no treatment, independently from congenital thrombophilic condition and with the exclusion of women affected by antiphospholipid syndrome [14].

AIM OF THE STUDY

The objective of this prospective pilot study was to assess the recurrence rate and severity of abnormal pregnancy outcome, excluding early pregnancy complications, in pregnant patients, without acquired thrombophilia, treated by prophylactic doses of LMWH, independently from their congenital thrombophilic condition.

MATERIALS AND METHODS

Design of the study

Starting in December 2001 to December 2008, we prospectively recruited a cohort of consecutive pregnant patients counseled for one or more previous APO at our interdisciplinary Centre for obstetrical immuno-haematolgy.

All eligible patients were treated by prophylactic doses of LMWH from the time of recruitment. The standard dose was 4250 U.I. die of enoxaparin. In two cases of coexisting decrease of fibrinogen and increase of D-dimer it was increased to 8500 UI until delivery.

When the study was commenced the prophylactic dose of LMWH for eligible patients had been a local standard of care in our interdisciplinary Center since 1998. In 2004, when the EMEA regulations for human research were accepted and enforced by the Italian law, this prospective observational study was communicated to the Ethical Committee and it was allowed to continue to its end with the same informed consent already in use which had been obtained by all patients. Patients with preeclampsia in their history were given also prophylactic low dose ASA at the time of recruitment. Each specific complication was treated according to local protocols, i.e. calcium-antagonist and beta-blockers for hypertension, corticoids for Hellp syndrome. Fetal growth restriction was monitored according to the TRUFFLE protocol [15].

Inclusion criteria for this study were one or more of the following complications in the previous index pregnancy: 1) positive history of intrauterine death (IUFD) after 20 weeks of gestation and before 37 weeks of gestation, associated with weight below the 10th 22 percentile for local standards (SGA) 2) fetal growth restriction (IUGR) 3) gestational hypertension (GH) based on the standard pressure measurements above 140/90 and preeclampsia (PE) when GH and 24 hours proteinuria above 300mg were recorded, 4) Hellp syndrome based on hemolysis, platelet count below 100.000 per ml and elevated liver enzymes, 5) clinical diagnosis of abruptio placentae. Early pregnancy loss was not considered an abnormal pregnancy outcome. Fetal growth restriction was defined as intrauterine assessment of restricted growth of the abdominal circumference according to local standards [16-17].

Exclusion criteria consisted of any of the following: gestational age at counseling >32 weeks of the current gestation; multiple gestation; a previous uneventful pregnancy; a previous pregnancy treated with LMWH or UFH; patients with clinical immune disease and acquired thrombophilia - Lupus Like Anticoagulant (LAC) or antiphospholipid syndrome (APL); patients with positive antinuclear, anti mitochondria, anti smooth muscle antibodies; postnatal or post-mortem diagnosis

of congenital fetal anomaly or fetal 1 infection; women of non- Caucasian ethnicity; alcohol or illicit drug use; early pregnancy loss was not consider an abnormal pregnancy outcome.

APO was grouped into two subsets: group 1 characterized by both maternal (GH, PE, HELLP, Abruptio) and fetal complications (IUGR and SGA with IUD), and group 2 with maternal complications only.

We could classify previous pregnancy based on the letter of hospital discharge, not on the original clinical record. This allowed us to define a reliable diagnosis of specific APOs, whereas it was impossible to go through a detailed classification of severity for each given complication. We introduced unequivocal clinical criteria to define a severe APO in the index pregnancy: any complication that led to an iatrogenic delivery $\leq$ 32 weeks of gestation. These same criteria were used to assess the overall severity of recurrence in the composite outcome of treated pregnancies.

Women were studied for congenital thrombophilic conditions: Leiden mutation of factor V, prothrombin G20210A mutation, MTHFR homo-zygotic mutation and homocysteinemia, protein C, protein S. These examinations were available throughout the whole study.

Additional more recent tests introduced during the study were not considered in this cohort.

For each patient we recorded age, gestational age at the time of recruitment and the main indices of severity: gestational age at delivery, mode of delivery and neonatal survival.

Follow-up was restricted to the early neonatal period or until discharge from the hospital of the mother and newborn.

The study was completed after more than one hundred and thirty eligible patients were treated. Compliance to treatment and follow-up was unexpectedly high given the severe obstetric history.

Statistics

The sample size of 120 patients was calculated by considering a 25% recurrence rate of severe APO and hypothesizing that LMWH could reduce recurrence by 50%. We calculated that 120 patients would be enough to detect a significant difference, for $\alpha = 0.05$, and a power of 98%.

Baseline demographic data were analyzed by parametric and non-parametric descriptive statistics as appropriate. Incidence and severity of APO in the index pregnancy and in pregnancies treated with LMWH were analyzed and compared in the whole cohort, by parametric and non-parametric tests. The primary outcome was the recurrence of any severe APO that led to iatrogenic delivery $\leq$h32 weeks of gestation. Relative risk (RR) was used to estimate the risk of recurrence of severe outcome in LMWH treated 1 pregnant patients. Chi Square test with Yates correction and Fisher exact tests were used as appropriate. In a second step the cohort was subdivided according to the presence or absence of any congenital thrombophilia, and recurrence rate and severity re-evaluated. A third

analysis was performed subdividing the cohort according to the gestational age at recruitment in order to observe the efficacy of timing of LMWH therapy. No missing data were observed in the limited items of retrospective information required.

RESULTS

One hundred and thirty three Caucasian patients were eligible for this prospective study. One hundred and twenty eight completed the study. Mean maternal age was 34 years (interquartile range 26-41). Parity was > 1 in 12 (9.3%) women. Median gestational age at initiation of LMWH therapy was 20 weeks (i.q. 12-27). Low dose ASA had been added to drug regimen in 45 patients with previous PE.

Figure 1 shows the maternal and feto-maternal APO in the index pregnancy and in the treated pregnancy. The overall recurrence rate in the treated pregnancy was 28%, and not significantly different in the two groups (p=n.s.). Details on cross over recurrences of APO from feto-maternal to maternal only and vice versa are reported in tables Ia and Ib. The recurrence of severe APO in all treated pregnancies, independently from maternal or feto13 maternal APO in the index pregnancy, was 4% (R.R. = 0.3, CI 95% 0.2-0.8; Chi square test with Yates correction =0.003). In the group with abnormal feto-maternal outcome, newborn weight was significantly greater even in the treated pregnancy with severe APOs than in severe APO at index pregnancy: 1090g (1035-1145) vs. 850g (535-1200), (P<0,01).

Neonatal mortality in the index pregnancy was 10%, no neonatal deaths occurred in the treated pregnancies.

Table Ia shows the details of feto-maternal complications in index pregnancies and their cross over recurrence in treated pregnancies. In treated pregnancies not a single case of intrauterine fetal death was observed versus 44 cases of fetal intrauterine death associated with S.G.A. in the index pregnancy. GH or PE associated with IUGR recurred in only one case out of 17 cases observed in the index pregnancy.

Severe cases delivered ≤ 32 weeks of gestation are flagged in the index pregnancy and in the treated pregnancy and reported both as absolute and overall relative figures.

The R.R. for recurrence of severe APO in treated pregnancies was 0.3 (CI .95 = 0.1 – 0.8; Fisher exact test = 0.001).

In this subset of patients with feto-maternal complications, 70 patients were treated ≤24 weeks of gestation. Median gestational age at LMWH therapy was 14 weeks (i.q. 10 – 18 completed weeks of gestation); recurrence rate of severe APO 3 of 70, overall recurrence rate was 20%. In 30 patients treatment was started ≥ 24 weeks of gestation. Median gestational age at LMWH therapy was 29 week (i.q. 27 - 31 completed weeks of gestation); recurrence rate of severe APO was 1 of 30, overall recurrence rate was 37%. The Fischer exact test for overall APO recurrence in these two subsets was <0,08).

Table Ib shows the incidence of maternal complication in the index 1 pregnancy and the treated pregnancy. We found no cases of Hellp syndrome in the treated pregnancy and a decreased recurrence in abruptio placentae and GH, PE.

Severe cases delivered ≤ 32 weeks of gestation are flagged in the index pregnancy and in the subsequent complicated pregnancy. The recurrence rate of severe cases was limited to one pregnant woman who developed a severe fetal growth restriction (980gr) The reduction of severe APO in treated pregnancies was not significantly different (Fisher exact test = 0.6).

The R.R. was not calculated for this small group that was not large enough to accommodate the hypothesis of the power calculation.

Fifty patients (39%) screened positive for congenital thrombophilia. Tables 2a and 2b show the recurrence rate and severity of maternal and feto-maternal complications in the index pregnancy and the treated pregnancy stratified for the presence or absence of known thrombophilic conditions, respectively. Severe APO recurred in 4% of treated pregnancies in both groups. The overall recurrence rate was identical (26%) in the thrombophilic group and non-thrombophilic group. Significant better outcome were observed for feto-maternal complications both for thrombophilic and non-thrombophilic patients in the pregnancy treated by LMWH. This different outcome was not observed for patients with only maternal complications.

DISCUSSION

In this prospective study we enrolled Caucasian pregnant women with previous severe abnormal pregnancy outcome (APO) without any previous uneventful pregnancy or pregnancies treated by LMWH. For the purposes of this study early pregnancy loss was not considered an abnormal pregnancy outcome. Enrollment was based on natural referral and as a consequence prophylactic LMWH was started in the vast majority of cases in the second trimester.

In this cohort, in which the index pregnancy was classified based on letter of hospital discharge, we flagged severe APO whenever any abnormal pregnancy outcome led to iatrogenic delivery ≤32 weeks of gestation. This severe complication was reported in 53% of feto-maternal APO and in 18% of maternal only APO. In these patients, treated in the present pregnancy with prophylactic doses of LMWH, the overall recurrence of severe outcome with iatrogenic deliveries ≤ 32 weeks of gestation decreased from 45% was 4%, the R.R. was 0.3 (CI 95% 0.2-0.8).

In 100 pregnant women fetal growth restriction was observed in the index pregnancy either as a marker of feto-placental disease associated with gestational hypertension, preeclampsia, HELLP, and abruption (37 cases), or as fetal condition caused by "ischemic placental disease" [18] without maternal clinical signs (63 cases). The severity of these latter cases referred to our Centre was underlined by the fact that more than fifty per cent of cases of growth restricted fetuses had ended in an intrauterine fetal death in the index pregnancy, and 53% were delivered before 32 weeks of gestation. In these treated pregnancies recruited for feto-maternal complications in the index pregnancy the recurrence of severe APO was 4% (R.R. = 0.3; CI .95% 0.1 – 0.8).

The initiation of LMWH before 24 weeks appeared to be more effective, but this study was not designed and powered enough to sort out this difference.

The 28 pregnancies with maternal APO only in the index pregnancy probably belong to a different pathological condition than those with feto-maternal APO. In fact, these latter patients were delivered significantly later in gestation, and with significantly better newborn weight than the group of patients with associated feto-maternal complications ($p<0001$). This occurred even though their final diagnosis had similar definitions under the present questionable codification of obstetrical disease. A non-significant lower recurrence rate was observed for severe APO in these treated pregnancies recruited for abnormal maternal outcome only.

The recurrence rate observed in 100 pregnancies treated by LMWH, based on severity and on composite maternal and fetal disease, and not only on the numbers of diseases as coded by present International Classification of Diseases, is very much lower than reported recurrence figures for severe APO in the first pregnancy both in small cohorts [19-20] and on large population based studies [18-21]. A strikingly reduction was observed in the recurrence of abruption. The possible

role of LMWH prophylaxis in reducing this severe APO has already been reported by the NOH-AP randomized trial 8 [14] in which treatment of pregnancies with previous abruption was started at the time of early pregnancy and resulted in a recurrence rate of 10 out of 80 cases in the LMWH treated pregnancies versus 25 out of 80 in non treated cases.

A limitation in our prospective non-randomized study is the possible additional role of ASA in patients with previous PE. In fact, 45 patients in our study had been treated with LMWH and ASA because of their history of PE. The appropriateness of this regime had been recently confirmed by a recent meta-analysis of Bujold [22] observed a significant reduction of the relative risk (RR=0,45) of recurrence in pregnant women treated by ASA before 16 weeks of gestation. We could only speculate that this reduction appears to be lower than the one we observed in our cohort in which PE was not the single most frequent disease both in index and in treated pregnancy. Indeed, only a prospective randomized trial could solve the relative weight of the two regimens.

Overall, when this cohort was stratified for the presence or absence of thrombophilia the recurrence rate of severe APO was 4% both in thrombophilic and non-thrombophilic pregnant women. This is very much in agreement with the figures of the small randomized study on LMWH prophylactic treatment reported by Rey [13] on non-thrombophilic pregnant women in whom the recurrence rate of severe APO in the treated arm was 5.5%. To make a complex problem simple we could say that this is in agreement with the statements of Rodger et al [7] that congenital thrombophilia per se has not been proven as a cause of placenta mediated pregnancy complications and LMWH might be beneficial in preventing placental damage based on its potential immune-modulation besides its known antithrombotic rule 30 [23].

This could be active not only in early gestation by promoting trophoblastic invasion [24] but all along placental development and ongoing function.

When both severe and all APO were considered altogether in the composite recurrence outcome analysis, we observed an overall recurrence rate of 25% and 29% for patients with feto-maternal and maternal complications in the index pregnancy, respectively. These findings lay well within the huge range reported in literature for observational studies. Even though our small cohort does not fit the criteria for a proper epidemiological comparison, when we consider our slightly lower percentage of recurrence versus comparable data reported by Likke 5 [21] (37.8% composite recurrence rate for preeclampsia occurring between 20 and 36 weeks) we should take into account the otherwise much higher recurrence expected in our cohort given the high prevalence of intrauterine fetal death [25] and for HELLP 7 [26], which altogether in the group of 100 pregnancies with feto-maternal complications summed up to 50 cases in the index pregnancy and was down to two cases only in the LMWH treated pregnancy.

A major growing body of evidence is suggesting that under the names of preeclampsia and gestational hypertension there are at least two diseases with different maternal vascular abnormalities 13 [27-28-29-30] and quite different placental pathology and fetal growth [31].

Recurrence rate in these two subsets should be investigated by different criteria rather than the present definition of these syndromes as per the CDI 9.

Gestational age at diagnosis had been frequently adopted to separate these different diseases, and the name early onset and late onset preeclampsia is now used in clinical settings. However, it is very likely that this time based criteria are not enough to separate these entities. Placental pathology, fetal growth and uterine Doppler waveform, or at least the two latter, should be included into this classification. To assess a proper figure of recurrence, Likke [21], Sibai [26-32] and Van Rijn [33] corrected the recurrence rate of pregnancies with "placental ischemic diseases" according to gestational age at diagnosis.

Another confounding factor in assessing the real recurrence of a condition, such as preeclampsia, IUGR or abruptio placentae, is that in the subsequent pregnancies these abnormal outcomes are interrelated. Ananth [18], on a large series of 154,810 patients, confirmed a crossover of preeclampsia, small for gestational age newborns and abruption from one pregnancy to the subsequent one. This composite outcome is mandatory to assess the true recurrence rate as confirmed by his larger series of 536,419. Both populations studies by Ananth [18], and Lykke [21] would have underestimated the recurrence rate of APO from 27.1% to 16.5%, if only preeclampsia were tracked in the subsequent pregnancy.

The finding that in our present cohort pregnancies recruited for previous maternal APO only, without clinical evidence of fetal growth restriction, did not show any significant difference between index and present pregnancy treated by LMWH in terms of gestational age and weight at birth, is in agreement with this criteria to separate early and late onset preeclampsia.

Beyond speculations, these findings suggest that women with 1 late onset gestational hypertension and preeclampsia at their first pregnancy should not be included in randomized studies on LMWH role for this could represent a useless recruitment of patients who are not likely to have any benefit by LMWH.

The strength of our findings is based on the strict criteria of recruitment, including homogenous ethnicity. Obviously strict monitoring and possibly better treatment regimen in our referral center and the strict collaboration between feto-maternal medicine specialists and immune-hematologists could have contributed to present results, yet given the state of clinical skills in the geographical area of recruitment where the index pregnancies were monitored and treated, we believe that the major difference was in fact determined by the usage of LMWH.

Major limitations are the non-randomized design of this pilot study and the gestational age at recruitment and initiation of therapy, based on the timing of referral. Early placentation was already been completed in most of the cases. However, this limitation together with the striking reduction of severe APO in recurrence should strongly induce to consider the possible role of LMWH during second wave of trophoblastic invasion and all along placental function, and not only from the very early stages of placental developmenT [14-34].

In conclusion, in this prospective study, prophylactic doses of LMWH after the first trimester reduced the recurrence rate of severe composite APO to 4%. These findings confirm that recurrence rate of APO in thses studies should consider the overall prevalence as well as severity of recurrence and the crossover of different APOs caused by placental ischemic disease. This recurrence rate observed in treated pregnancies was in close agreement with the small but significant randomized trials [18-19] so far published on LMWH prophylaxis forprevention of APO, and is independent from inherited thrombophilic conditions.

The less significant role of LMWH in reducing the severity and overall recurrence of APOs occurring late in pregnancy or without fetal growth restriction addresses the need of overcoming the bias induced into any clinical study by present definition of these syndromes as per the CDI 9 which groups under the same heading really different diseases.

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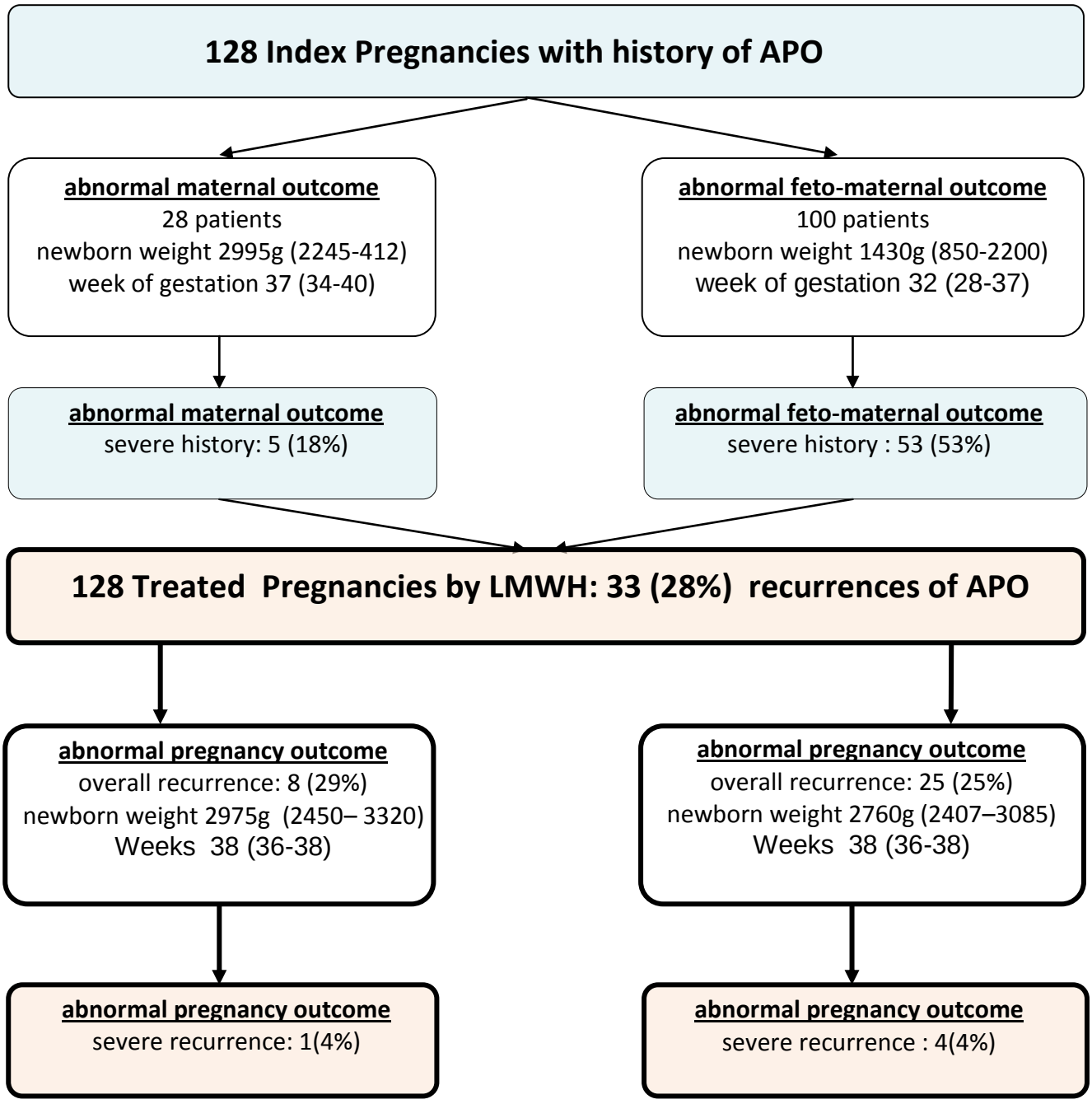
Figure 1

Flow chart of pregnancy outcome of index pregnancies and subsequent pregnancies treated by prophylactic doses of LMWH

Thin boxes: Index pregnancies. Gestational age and newborn weight (median and i.q.) were significantly worse in the feto-maternal than in the maternal APO group ($p<0001$). The prevalence of severe APO was significantly higher in the feto-maternal APO group ($p<0.001$)

Thick boxes: treated pregnancies. Feto-maternal APO: gestational age at delivery and newborn weight (median and i.q.) in the treated pregnancy were highly significantly better than in the index pregnancy (Mann-Whitney test $p<0.001$). Maternal APO: gestational age at delivery and newborn weight in the treated pregnancy were not significantly better than in the index pregnancy (Mann-Whitney test p<n.s.). The severe and the overall recurrence rate of APO were not significantly different in the two groups (p=n.s.).

Figure 1


128 Index Pregnancies with history of APO
abnormal maternal outcome
28 patients
newborn weight 2995g (2245-412)
week of gestation 37 (34-40)
abnormal feto-maternal outcome
100 patients
newborn weight 1430g (850-2200)
week of gestation 32 (28-37)
abnormal maternal outcome
severe history: 5 (18%)
abnormal feto-maternal outcome
severe history : 53 (53%)
128 Treated Pregnancies by LMWH: 33 (28%) recurrences of APO
abnormal pregnancy outcome
overall recurrence: 8 (29%)
newborn weight 2975g (2450– 3320)
Weeks 38 (36-38)
abnormal pregnancy outcome
overall recurrence: 25 (25%)
newborn weight 2760g (2407–3085)
Weeks 38 (36-38)
abnormal pregnancy outcome
severe recurrence: 1(4%)
abnormal pregnancy outcome
severe recurrence : 4(4%)

Tables

Table Ia. Analytical description of feto-maternal complications in the index pregnancy and in the treated pregnancy. Pregnancies delivered ≤32 weeks of gestation were flagged as severe abnormal pregnancy outcome (APO).

index pregnancy	All APO	Severe APO	treated pregnancy	All APO	Severe APO
FETO-MATERNAL COMPLICATIONS	100	53	FETO-MATERNAL COMPLICATIONS	15	1
GH – PE - IUGR	17	5	GH – PE - IUGR	1	
GH – PE - IUGR -IUFD	6	5		-	
ABRUPTIO IUGR	6	5		-	
HELLP IUGR	6	3	HELLP IUGR	1	
HELLP IUGR IUFD	2	2		-	
IUGR	27 *	3	IUGR	13	1
SGA -IUFD	36	30		-	
			MATERNAL COMPLICATIONS	10	3
			GH	3	
			PE	2	
			HELLP	1	
			ABRUPTIO	4	3
TOTAL (%)	100	53 (53%)		25 (25%)	4(4%)

*the median weight of these cases was 1754,4 +/- 991 g and the median gestational age at delivery was 34 (r.i. 36-38.5)

Table Ib. Analytical description of maternal complications in the index pregnancy and complications in the treated pregnancy. Pregnancies delivered ≤32 weeks of gestation were flagged as severe abnormal pregnancy outcome (APO).

index pregnancy	All APO	Severe APO	treated pregnancy	All APO	Severe APO
MATERNAL COMPLICATIONS	28	5	MATERNAL COMPLICATIONS	2	0
GH	5				
PREECLAMPSIA	9	2	PREECLAMPSIA	1	
HELLP	5	1			
ABRUPTIO	9	2	ABRUPTIO	1	
			FETO-MATERNAL COMPLICATIONS	6	1
			IUGR	6	1
TOTAL (%)	28	5 (18%)		8 (28%)	1 (4%)

Legend a) and b) panels.

APO= abnormal pregnancy outcome, excluded first trimester complications; Severe APO = abnormal pregnancy outcome which led to iatrogenic delivery before 32 completed weeks of gestation; GH = gestational hypertension; PE= preeclampsia; IUGR= intrauterine growth restriction; IUFD= intrauterine fetal death; ABRUPTIO= abruption placenta; HELLP = help syndrome; SGA= small for gestational age.

Table IIA. Fifty inherited thrombophilic patients: Analytical description of rate and severity of recurrence of APO (*). Pregnancies delivered ≤32 weeks of gestation were flagged as severe abnormal pregnancy outcome (APO). (median and interquartile range in brackets)

Index pregnancy	All APO	Severe APO	treated pregnancy	All APO	Severe APO	P <
FETO-MATERNAL COMPLICATIONS	41	16	FETO-MATERNAL COMPLICATIONS	8	1	
Weeks at delivery	35 (30-38)		Weeks at delivery	38 (36,7-38,25)		0,04
Weight (g.)	1600 g (958-2300)		Weight (g.)	2100 g (1990-2395)		0,08
MATERNAL COMPLICATIONS	9	0	MATERNAL COMPLICATIONS	5	1	
Weeks at delivery	38 (36-40)		Weeks at delivery	37 (35-38)		0,41
Weight (g.)	3000 g (2440-3200)		Weight (g.)	2870 g (2290-3180)		0,56
Total	50(100%)	16(32%)		13 (26%)	2 (4%)	

(*) 19 women (15,6%) were eterozigotic for V Leiden mutation, 19 women (15,6%) were eterozigotic for G20210A prothrombin mutation, 13 (10,1%) were homozigotic for MTHFR mutation, two with hyperhomicisteinemia, and 11(8,6%) patients had more than one mutation. Not a single case of S and C deficiency was recorded in this cohort.

Table IIB. Non thrombophilic patients: analytical description of rate and severity of recurrence in . Pregnancies delivered ≤32 weeks of gestation were flagged as severe abnormal pregnancy outcome (APO). (median and interquartile range in brackets)

index pregnancy	All APO	Severe APO	treated pregnancy	All APO	Severe APO	P <
FETO-MATERNAL COMPLICATIONS	59	36	FETO-MATERNAL COMPLICATIONS	13	1	
Weeks at delivery	31 (28-36)		Weeks at delivery	36 (35-37)		0,03
Weight (g.)	1295 g (798-2015)		Weight (g.)	2180 g (1890-2450)		0,001
MATERNAL COMPLICATIONS	19	5	MATERNAL COMPLICATIONS	7	2	
Weeks at delivery	36 (33-39)		Weeks at delivery	37 (33,5-38)		0,97
Weight (g.)	2990 g (1945-3415)		Weight (g.)	2650 g (2060-3085)		0,72
total	78(100%)	41(53%)		20 (26%)	3 (4%)	

Legend a) and b) panels.

APO= abnormal pregnancy outcome, excluded first trimester complications; Severe APO = abnormal pregnancy outcome which led to iatrogenic delivery before 32 completed weeks of gestation;