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The vitamin E-binding protein afamin increases in maternal serum during pregnancy

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ABSTRACT

Background: Afamin is a liver-derived plasma glycoprotein with vitamin E-binding properties and a putative function in fertility. This study evaluated serum afamin concentrations during and postpartum to uncomplicated pregnancies and investigated a potential association between afamin concentrations and pregnancy outcome.
Methods: Afamin serum concentrations were measured in women with uncomplicated pregnancies in a retrospective cohort (n = 466) at different gestational ages and a prospective observational study (n = 76) in the first, second and third trimester. Furthermore, afamin was determined in the first trimester in a cross-sectional pilot study including women with preeclampsia (PE), pregnancy-induced hypertension (PIH) and women without pregnancy complications (n = 13 each). Finally, expression of afamin was investigated in human placental tissue by RT-PCR and immunohistochemistry.
Results: Afamin concentrations increased linearly almost two-fold during pregnancy in both retrospective and prospective studies in women without pregnancy complications with median afamin serum concentrations of 61.9 mg/l, 79.6 mg/l, and 98.6 mg/l in the first, second, and third trimester, respectively. After delivery, median afamin concentrations decreased to baseline values of 54.6 mg/l. In the pilot study with pregnancy complications, women with PE displayed significantly higher median afamin concentrations than did women with uncomplicated pregnancy (70.0 mg/l vs. 55.4 mg/l, P = 0.007). Expression analyses revealed no placental afamin expression at either mRNA or protein level in uncomplicated pregnancy.
Conclusion: A linear increase in the maternally expressed glycoprotein afamin during pregnancy may serve as basic reference for subsequent investigations of afamin in pregnancy-related disorders.

1. Introduction

Pregnancy is generally characterized by an increased generation of reactive oxygen species (ROS). This is especially true for the placenta, where mitochondrial activity and production of free superoxide radicals increase ROS quantity. It is typically accompanied by reduced levels of antioxidants [1]. Imbalanced or poorly controlled oxidative stress related to dysregulated trophoblast development may lead to pregnancy-associated complications, such as hypertensive disorders or fetal growth restriction [2]. With their ability to stabilize reactive free radicals, antioxidant vitamins act as the first line of defense against free radical attack and lipid peroxidation. Vitamin E is the major lipophilic antioxidant nutrient in the early stages of life from the time of conception, during pregnancy and through the postnatal development of the infant [3,4]. Mechanisms of its uptake by placenta and mammary gland most likely depend on lipoprotein receptors since most vitamin E in human plasma is transported by lipoproteins.

The plasma glycoprotein afamin has been previously identified [5] and described as an alternative carrier protein for vitamin E [6,7] in extravascular fluids, such as follicular fluid, known for its reduced

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lipoprotein content [8], suggesting a role of afamin in female fertility. Afamin belongs to the albumin gene family, is primarily expressed in liver and is secreted into blood with subsequent distribution to the respective extravascular fluids [9].

In order to better understand the role of afamin in human fertility we measured serum concentrations of afamin by ELISA [10] at various gestational ages in women with uncomplicated pregnancies. Afamin concentrations were correlated with those of recognized pregnancy markers such as β-human chorionic gonadotrophin (hCG+β), human placental lactogen (hPL) and free estriol. The marker proteins hCG+β and hPL are synthesized by the human placenta while the steroid estriol is a product of the materno-placento-fetal unit, thus reflecting both feto-placental growth and development [11]. We then evaluated the results of the retrospective study in a second, prospective observational study in samples obtained at pre-specified time intervals from women with uncomplicated pregnancies. Based on this systematic study of afamin in uncomplicated pregnancy, afamin was finally also measured in a pilot study of women diagnosed with pregnancy complications, such as preeclampsia (PE) and pregnancy-induced hypertension (PIH), with the aim of determining the potential of afamin as an early diagnostic or predictive marker for those conditions.

2. Materials and Methods

2.1. Subjects

The present multicenter study was performed by investigating several cohorts with different study designs.

The first, retrospective cohort study group comprised 466 consecutive healthy pregnant women undergoing routine perinatal screening between February and August 2006 at the Department of Gynecology and Obstetrics of Innsbruck Medical University, Austria.

The second, prospective observational study group comprised 76 healthy pregnant women recruited between April 2007 and November 2008 at the Department of Gynecology and Obstetrics at the Medical University of Graz, Austria. Three blood samples were taken at gestational weeks 12–13, 24–25 and 35–36, representing the first, second and third trimester of pregnancy, respectively. Blood was also collected postpartum from 17 (22%) participants of this study group 43 $\pm$ 7 days after delivery. At all times of blood collection, women in both study groups were free of any pregnancy-associated complications. Only women with uncomplicated singleton pregnancies carried to term were eligible for study participation.

A third, cross-sectional study group was recruited at the Department of Obstetrics and Gynecology, Danube Hospital/SMZ-Ost, Vienna, Austria and comprised 13 women diagnosed with PE, 13 women with PIH and 13 healthy pregnant women who served as controls. Women with pregnancy complications and uncomplicated pregnancies were matched for maternal and gestational age, gravidity and BMI; their blood was obtained at the occasion of "first trimester screening" between gestational weeks 11 and 14. Eight women with PE delivered their baby at gestational week ≥37, three at week <37 and two at week <35. PE was defined according to the classification of the International Society for the Study of Hypertension in Pregnancy (ISSHP) [12].

All study participants were Caucasian. The studies were approved by the internal review boards and the ethics committees of the Medical Universities of Innsbruck and Graz and the City of Vienna, in adherence with the Declaration of Helsinki; signed informed consent for participating in the study was obtained from all participants.

2.2. Analysis of parameters in serum samples

Blood was collected from all study participants after overnight fasting and serum was obtained by low-speed centrifugation. Serum aliquots were frozen and kept at −70 °C until analysis.

Concentrations of afamin were measured with a previously described double-antibody sandwich ELISA test, using a biotinylated affinity-purified polyclonal antibody for binding to streptavidin-coated microtiter plates and the peroxidase-conjugated monoclonal antibody N13 for detection. The intra-assay and inter-assay coefficients of variation were 3.3% and 6.2%, respectively, at a mean afamin concentration of 73 mg/l [10].

Serum concentrations of free (unconjugated) estriol were measured by competitive enzyme immunoassay; human placental lactogen (hPL) was measured by sandwich ELISA (DRG Instruments, Marburg, Germany). Human chorionic gonadotropin (hCG) was measured by sandwich immunoassay on the Modular Analytics Platform E170 (Roche Diagnostics, Mannheim, Germany). This assay quantifies the intact hCG molecule plus the free β subunit of hCG and is therefore referred to as hCG+β.

2.3. Afamin tissue expression analysis

Term and pre-term placental tissues from women with uncomplicated pregnancies were obtained from the Department of Gynecology and Obstetrics of the Medical University of Graz, Austria. First-trimester placental tissue was obtained after induced pregnancy termination for social, non-medical reasons.

Semi-quantitative RT-PCR was performed on RNA extracted from human first trimester and term placental tissue (n = 5 each) using six different afamin primers [13], with 36 cycles, annealing temperature 55 °C and 100 ng pooled total RNA applied for each reaction. Human ribosomal protein L0 (RPL0) served as endogenous control. Immunohistochemistry was performed on paraffin-embedded formaldehyde-fixed sections of human placental tissue (first-trimester and term, n = 5 each) using two different affinity-purified polyclonal anti-afamin antibodies (#2132 and #1055-4) raised in rabbits in our laboratory after immunization with native afamin, purified from human plasma [6]. Sections from human kidney known for afamin expression (see www.proteinatlas.org) served as positive controls, sections incubated without primary antibodies served as negative controls.

2.4. Statistical analyses

Log base 2-transformed serum concentrations of afamin, free estriol, hCG+β and hPL were compared across trimesters using ANalysis Of VAriance (ANOVA). Spearman's correlations between pairs of markers were computed for each trimester and additionally, on the basis of detrended residuals, by subtracting the ordinary least squares fitted mean from all observations, and then averaging over a four-week moving window with a step width of one week. Normal linear mixed models were used to model longitudinal trajectories of individual log base 2-transformed concentrations of afamin separately for the retrospective cohort and prospective observational studies according to gestational age, accounting for within-patient correlation (random effects) and influence of women's age, parity, birth weight of the newborn (Ponderal Index, PI), and body mass index (BMI) of the mother before onset of pregnancy (fixed effects). The Bayesian Information Criterion (BIC) was used to select the optimal mean time trajectory, namely logarithmic or quadratic, and fixed effects. All models contained random intercepts; additional random effects, such as random slopes, were tested using likelihood ratio tests.

Comparisons of afamin between women without and with pregnancy complications (i.e. PE and PIH) were performed with the Wilcoxon test and the non-parametric Mann–Whitney U test, as appropriate. Obtained p values were not adjusted for multiple comparisons and are therefore descriptive only.

All data were statistically analyzed with the SPSS 13.0 software (SPSS Inc.), the R-2.14.2 package and the MedCalc 12.7.0.0 package (MedCalc Software).

3. Results

3.1. Patient characteristics

Table 1 shows anthropometric characteristics of participating pregnant women with uncomplicated pregnancies. Their age and gestational age at delivery, pre-pregnancy BMI and Ponderal Index (PI) of newborns were comparable. In the retrospective study group, blood samples were acquired at different gestational ages; participants from the prospective study group donated three samples at pre-specified time points during pregnancy (see methods section).

3.2. Afamin in the retrospective study group with uncomplicated pregnancy

Median concentrations of afamin, free estriol, hPL and hCG+β for each trimester of pregnancy for the retrospective cohort study are listed in Table 2. Individual and population mean afamin curves over the course of pregnancy indicate afamin changes during the three trimesters of pregnancy (all P values <0.0001, Table 2, Fig. 1A–D). Residual correlations after adjusting for the mean changes over weeks of pregnancy between all markers were nearly negligible: between afamin and free estriol, −0.01; afamin and hPL, −0.09; afamin and hCG+β −0.03; free estriol and hPL, 0.37; free estriol and hCG+β −0.20; hPL and hCG+β 0.07. However, when evaluated separately for each trimester, afamin concentrations correlated significantly with all three parameters in the second, but not in the first and third trimesters.

Unlike the other known markers of pregnancy that exhibit nonlinear trajectories over the course of pregnancy, afamin showed a consistent linear increase during uncomplicated pregnancy with an average increase of 2.17% (95% CI = 2.03% to 2.31%) per week of pregnancy resulting in an approximate-doubling of extrapolated average of afamin values by the end of pregnancy (Fig. 1A). Specifically, the linear mixed effects model for the log base 2-transformed afamin course contained an intercept (estimate = 5.65, SE = 0.05, P < 0.0001) and slope for week of pregnancy (estimate = 0.031, SE = 0.001, P < 0.0001). There was significant random variation of afamin concentrations, both at the start and during the course of pregnancy (P < 0.0001).

In contrast to afamin, hCG + β showed a sharp decline over the first 20 weeks of pregnancy before reaching a plateau and slightly increasing again towards delivery at term (Fig. 1B). There was significant woman-to-woman variability in the time course (P = 0.0003). Of all transformations tested to describe the mean trajectory over weeks of pregnancy, a model containing an intercept term (estimate = 20.13, SE = 0.25, P < 0.0001), slope for week (estimate = −0.51, SE = 0.02, P < 0.0001), and a quadratic term for week (estimate = 0.009, SE = 0.0005, P < 0.0001) provided the best fit.

Free estriol and hPL followed similar trajectories during the course of pregnancy, steeply rising over the first one to two trimesters and continuing to rise gradually during the third trimester (Fig. 1C + D). There was significant woman-to-woman variability in the time course for hPL (P < 0.0001) and free estriol (P < 0.0001). The mean trajectory for free estriol was described by an intercept (estimate = −6.63, SE = 0.25, P = <0.0001), and slope for the logarithm of time (estimate = 3.77, SE = 0.08, P < 0.0001); for hPL the mean trajectory was described by an intercept (estimate = −19.56, SE = 0.82, P < 0.0001), slope for time (estimate = −0.17, SE = 0.02, P < 0.0001), and slope for logarithm of time (estimate = 7.80, SE = 0.39, P < 0.0001).

3.3. Afamin in the prospective study group with uncomplicated pregnancy

When comparing afamin concentrations in the prospective study of 76 women with uncomplicated pregnancies throughout pregnancy, median afamin serum concentrations were 61.9 mg/l (25%, 75%-ile 54.8–69.2 mg/l) in the first trimester, 79.6 mg/l (25%, 75%-ile 68.9–87.2 mg/l; Wilcoxon test, p <0.001 for comparison with afamin in the first trimester) in the second trimester, and 98.6 mg/l (25%, 75%-ile 89.1–107.5 mg/l; Wilcoxon test, p <0.001 for comparison with afamin in the first trimester; and Wilcoxon test, p <0.001 for comparison with afamin in the second trimester) in the third trimester of pregnancy (Fig. 2).

In 17 women with afamin values available also after delivery, afamin concentrations dropped to pre-pregnancy values (median 54.6 mg/l, 25%, 75%-ile 49.1-60.5 mg/l, range 41.8–70.6 mg/l).

3.4. Afamin in pregnancy complications

In the pilot cross-sectional study, first-trimester median serum concentrations of afamin (25%, 75%-ile) in pregnant women suffering from PE were found to be significantly higher than in pregnant healthy controls with the same gestational age (70.0 (68.0, 82.1) mg/l vs. 55.4 (50.8, 73.0) mg/l, P = 0.007). Women with PIH had intermediate afamin concentrations of 69.8 (61.2, 75.8) mg/l, P = 0.07 (Fig. 3).

3.5. Expression analysis

Afamin expression was investigated in first-trimester or term placental tissues at the mRNA and the protein level (Fig. 4A, B). No afamin mRNA expression was observed whereas the positive control showed a

Table 1
Participant characteristics at onset of uncomplicated pregnancy.

Characteristic		Retrospective cohort study (n = 466)	Prospective observational study (n = 76)
Age (yrs)	Median	28	32
	(25%, 75%-ile)	(24, 32)	(29, 35)
	Range	19, 44	19, 42
Parity N (%)	Nulliparous	219 (47)	38 (50)
	Multiparous	247 (53)	38 (50)
Gestational age at delivery (weeks)	Median	39	39
	(25%, 75%-ile)	(38, 40)	(39, 41)
	Range	25, 42	36, 42
BMI of women before pregnancy (kg/m^2)	Median	23.5	21.2
	(25%, 75%-ile)	(21.2, 27.1)	(19.9, 23.4)
	Range	14.6, 55.5	17.8, 34.0
PI of newborns (kg/m^3)	Median	26.4	25.1
	(25%, 75%-ile)	(25.0, 28.4)	(24.1, 27.0)
	Range	17.1, 34.5	20.6, 31.4

BMI, body mass index; PI, Ponderal Index.

Table 2
Biomarker characteristics by trimester of uncomplicated pregnancy (retrospective cohort study, n = 466, N = 659).

Biomarker		First Trimester (Weeks 1–12) N = 120	Second Trimester (Weeks 13–28) N = 279	Third Trimester (Weeks ≥29) N = 260	*P* value
Afamin (mg/l)	Median (25%, 75%-ile)	65.1 (52.5, 82.7)	87.8 (73.4, 98.8)	103.6 (93.0, 118.3)	<0.0001
Free estriol (ng/dl)	Median (25%, 75%-ile)	4.4 (2.8, 6.1)	44.3 (29.7, 65.6)	107.3 (72.8, 156.8)	<0.0001
hPL (mg/l)	Median (25%, 75%-ile)	0.1 (0.1, 0.2)	2.2 (1.3, 2.8)	4.1 (3.2, 5)	<0.0001
hCG+β (U/l)	Median (25%, 75%-ile)	74,429 (40,874, 98,989)	9533 (5309, 20,897)	12,654 (5534, 22,284)	<0.0001

n denotes number of patients, N denotes number of samples investigated.

clear signal. The same result was obtained with immunostaining: there was no staining signal found in placental tissue whereas kidney tissue showed a clear positive staining.

4. Discussion

This study demonstrates a nearly two-fold increase in serum concentrations of the vitamin E-binding protein afamin during the course of uncomplicated pregnancy. The reason for steadily increasing afamin concentrations in maternal blood during pregnancy is unclear. It is tempting to speculate that afamin rises during pregnancy due to changing hormonal status and subsequent hormonal regulation of the afamin gene expression in human liver. A comparable mechanism has been reported for hormonal regulation (mostly estrogen-induced) of hepatic synthesis of lipids and lipoproteins leading to physiological hyperlipidemia during gestation [14].

In contrast to the steady linear increase in afamin throughout pregnancy, time courses of hPL, hCG and free estriol followed non-linear trajectories in accordance with earlier observations [15,16]. hCG+β and hPL are synthesized by the placenta, while estriol is a product of the materno-placento-fetal unit, thus reflecting both feto-placental growth and development [11].

Placental tissue expression experiments at both the mRNA and the protein level revealed no placental expression of afamin suggesting a maternal origin of increasing circulating afamin concentrations during pregnancy. Theoretically, the increasing concentration of afamin during pregnancy could also be due to embryonal and fetal expression of afamin. However, reports about lacking expression of afamin (also named "alpha-albumin") in the fetal rat liver [17] and human

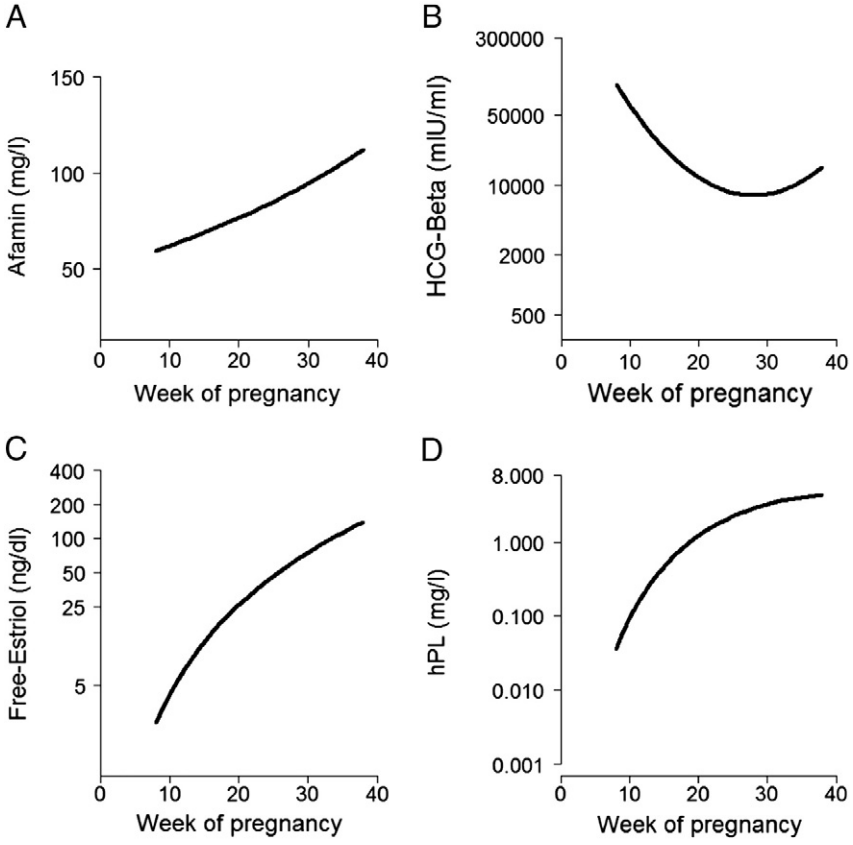


Fig. 1. Serum concentrations of afamin and established pregnancy biomarkers during uncomplicated pregnancy in a retrospective cohort study (n = 466). Trajectories are displayed by overall means for A) afamin, B) hCG+β, C) free estriol, D) hPL.

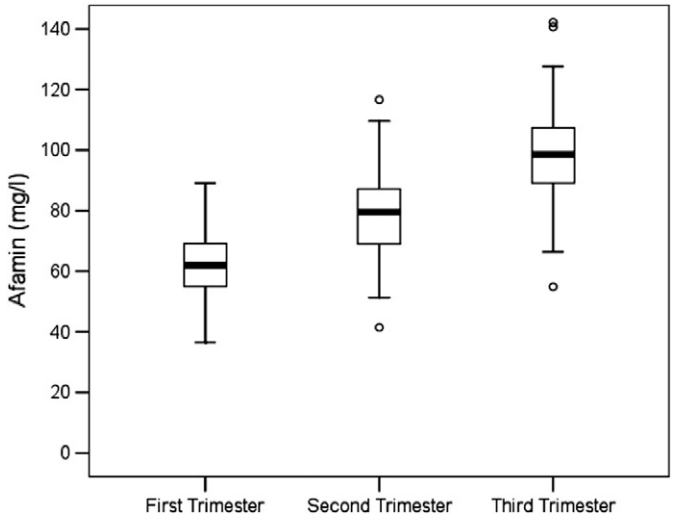


Fig. 2. Afamin in a prospective study group with uncomplicated pregnancy (n = 76). Box plots display median concentrations and bars indicating the 25% and 75%-ile of afamin serum concentrations in the first, second and third trimester of pregnancy at pre-specified time points. Afamin concentrations were 61.9 mg/l in the first trimester, 79.6 mg/l (Wilcoxon test, $p < 0.001$ for comparison with afamin in the first trimester) in the second trimester, and 98.6 mg/l (Wilcoxon test, $p < 0.001$ for comparison with afamin in the first trimester; and Wilcoxon test, $p < 0.001$ for comparison with afamin in the second trimester)) in the third trimester of pregnancy.

embryogenesis during weeks 4 to 9 [18] are not in favor of prenatal afamin expression and therefore most likely reflect a maternal origin of increasing afamin concentrations during pregnancy. Interestingly, this observation stands in clear contrast to established pregnancy-related parameters, including estriol, hPL, hCG and adrenomedullin that are all synthesized by the human placenta [11,19]. Adrenomedullin is a vasorelaxing peptide involved in blood flow regulation. Similar to afamin, its plasma concentrations increase linearly during pregnancy and correlate significantly with placental hormones, such as hPL [19,20].

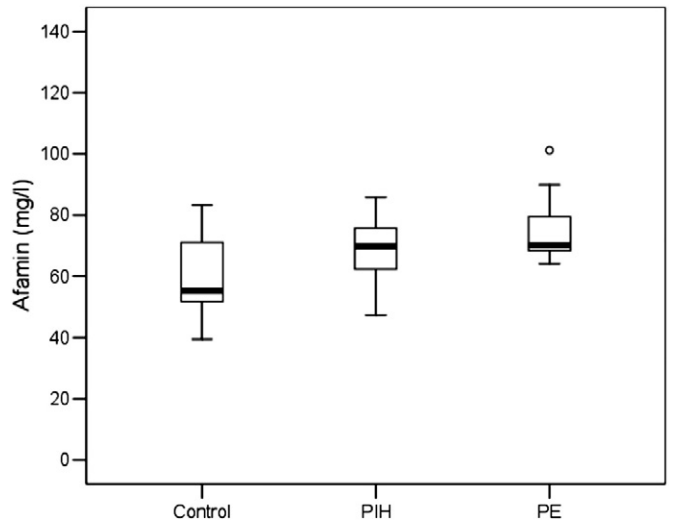


Fig. 3. Afamin concentrations in pregnancy complications (cross-sectional). Box plots of afamin serum concentrations in first trimester of pregnancy from healthy controls (n = 13) and patients diagnosed with PIH (n = 13) and PE (n = 13). Median serum concentrations of afamin in pregnant women suffering from PE were found to be significantly higher than in pregnant healthy controls with the same gestational age (70.0 mg/l vs. 55.4 mg/l, P = 0.007). Women with PIH had intermediate afamin concentrations of 69.8 mg/l, P = 0.07.

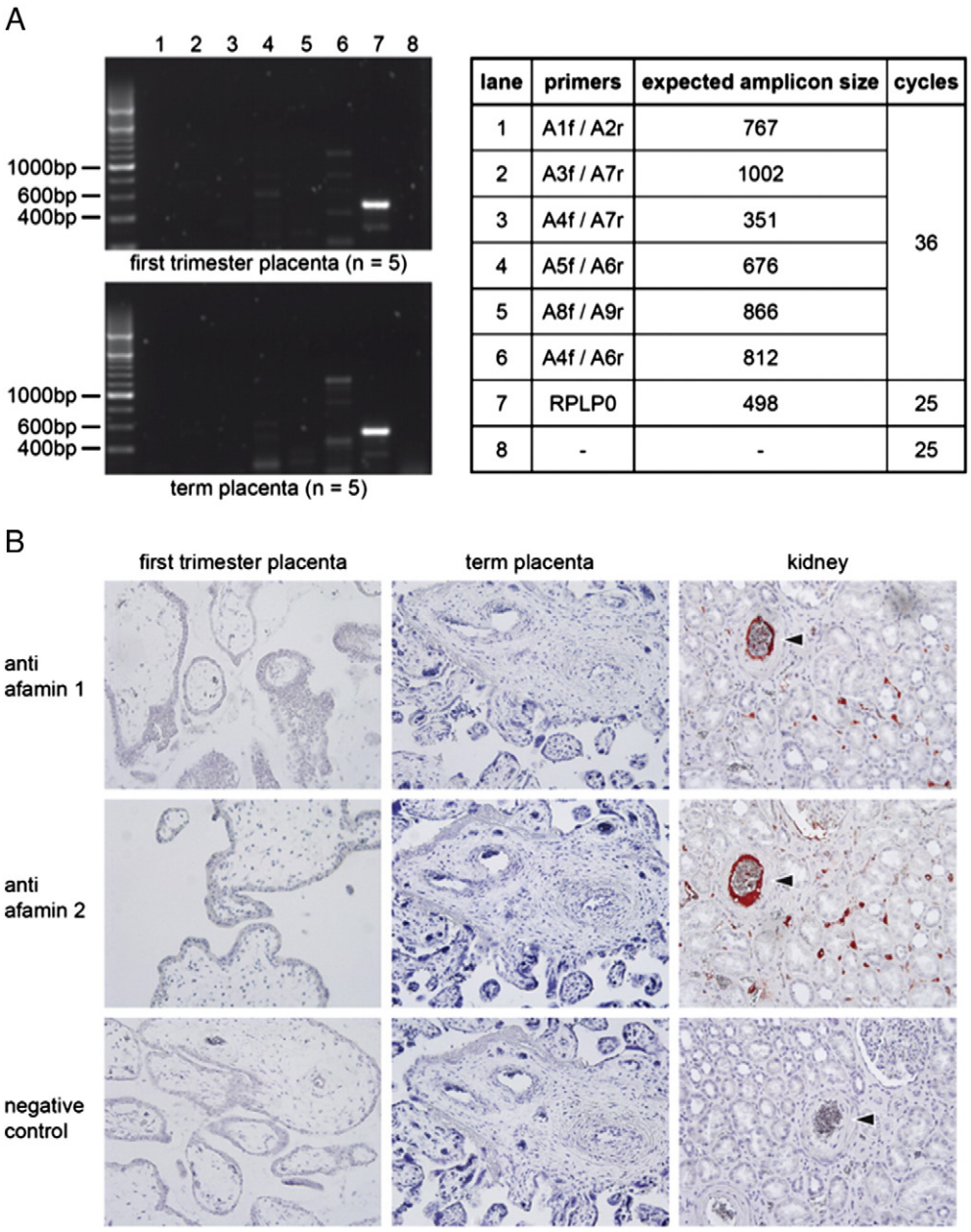
Women destined to develop hypertensive pregnancy complications such as PE or PIH showed higher serum concentrations of afamin in the first trimester than did gestational age-matched healthy pregnant controls. The difference between PE and healthy controls was significant, whereas PIH and controls differed only with borderline significance, possibly due to the small (pilot) study size. Further prospective studies with substantially larger patient groups will be necessary to evaluate afamin as potential early marker for PE and related pregnancy disorders.

Hypertensive disorders of pregnancy affect up to 8% of all gestations and remain major causes of maternal and neonatal mortality and morbidity worldwide [21]. Preeclampsia (PE) is a multisystem pregnancy disorder that complicates up to 3% of pregnancies depending on the developmental stage of the respective world region [22–24]. The precise etiology is not fully understood; one undisputed origin lies in the placenta. Impaired placental development leads to increased tissue oxidative stress and placental apoptosis and necrosis, resulting in the subsequent release of subcellular fragments causing a systemic effect in the mother [25,26].

In PE, cytokines and ROS released from the mal-developed placenta trigger a systemic inflammatory and oxidative state [27]. The maternal endothelium as well as the placenta also overexpresses anti-angiogenic factors that inhibit the normal function of pregnancy-related pro-angiogenic factors. The combination of these factors is believed to underlie a systemic endothelial dysfunction that is consistently found in the mother during symptomatic PE. Likewise, metabolic complications including insulin resistance, coagulation defects and hyperlipidemia contribute to an increased risk for cardiovascular diseases. Therefore, PE and related pregnancy complications have been previously discussed as the "metabolic syndrome of pregnancy" [28]. Although reversible after delivery, women suffering from such pregnancy disorders carry increased additive risks for future cardiovascular diseases. Our findings raise the possibility of associations between afamin and components of the metabolic syndrome also in the general population.

Despite intensive research, risk estimation for developing PE prior to the onset of symptoms did not improve significantly until very recently [29]. As previously reviewed [30], the heterogeneous nature of PE calls for a combination of independent biomarkers, each representing a pathophysiological process, to provide suitable early diagnostic power for predicting PE. Such markers would contribute substantially to identifying high-risk women in need of closer supervision in secondary care. Additionally, there is now evidence from a recent meta-analysis that the prophylactic use of low-dose aspirin may reduce the incidence of PE by about 50%, provided that treatment starts before the 16th week of pregnancy [31]. These findings highly reinforce the need for early identification of women at risk for developing PE during pregnancy with the objective of implementing therapeutic interventions to improve perinatal and maternal outcome. Indeed, very recent screening studies of first-trimester samples of large cohorts of uncomplicated and pathological pregnancies revealed detection rates for developing early and late PE of 90% and 60%, respectively, when maternal factors, biophysical and biochemical markers were combined [23,32]. Biochemical plasma markers, thought to be involved in placentation, include soluble endoglin, placental protein 13 (PP-13) [33] and pregnancy-associated plasma protein A (PAPP-A). Although these very recent substantial achievements have led to promising first-trimester risk assessments for developing PE, further large clinical studies including additional biomarkers are needed to translate the current marker panel into routine clinical practice.

The physiological function of afamin is largely unknown. Although reduced afamin plasma concentrations have been described in patients diagnosed with ovarian cancer, the evidence for a causal relation is lacking [34,35]. The previously reported abundance of afamin in human follicle fluid suggests the importance of afamin for oocyte development, maturation and hence fertility in general, most likely due to its vitamin E-carrying property [6]. Recently, afamin was identified as a potential biomarker for trisomy 21 by proteomic comparative analysis of plasma



lane	primers	expected amplicon size	cycles
1	A1f / A2r	767	36
2	A3f / A7r	1002	
3	A4f / A7r	351	
4	A5f / A6r	676	
5	A8f / A9r	866	
6	A4f / A6r	812	
7	RPLP0	498	25
8	-	-	25

Fig. 4. Afamin expression in human placenta. Expression analysis of afamin in human first-trimester and term placenta (n = 5, each). A, RT-PCR of RNA extracted from placental tissue; used primers are described in inserted table [13]. B, immunohistochemistry of human placental tissue using two different polyclonal anti-afamin antibodies (anti-afamin 1, 2). Sections from human kidney served as positive controls, sections incubated without primary antibodies as negative controls. Arrowheads point to glomerulus. Magnification 360x.

from pregnant women [36]. However, this finding was not confirmed in a later study comparing 25 women pregnant with trisomy-affected babies and 50 euploid healthy pregnancies [36].

In conclusion, findings of a linear, nearly two-fold increase in the maternally expressed glycoprotein afamin during pregnancy have been used as basic reference for subsequent investigations of afamin in pregnancy-related disorders and suggest a potential for afamin to serve as a predictive marker for such conditions. However, further prospectively planned and adequately powered studies are needed to confirm the potential role of afamin as marker for pregnancy complications.

Conflict of interest statement

H.D. is owner and shareholder of Vitateq Biotechnology GmbH, Innsbruck, Austria, a spin-off biotech company from Innsbruck Medical University, holding several patents related to research described in this article. All other coauthors report no conflict of interest.

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