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Alpha-fetoprotein (AFP) — new aspects of a well-known marker in perinatology

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ABSTRACT

Alpha-fetoprotein (AFP) is a serum protein, which is characteristic of the fetal development period and a well-known oncological marker. The predominance of AFP among serum proteins is common in fetal life, whereas after birthing its functions are gradually taken over by albumins. An understanding of the mechanism of AFP transfer between fetus and mother has led to the development of screening tests for identifying neural tube defects and Down's syndrome. Currently, the knowledge on patophysiology and the possible importance of AFP in perinatology and fetal medicine extends far beyond those 2 disease states. Throughout the 50 years of research on AFP, there has been dynamic progress of diagnostic techniques, from the qualitative ones that are used solely for scientific studies to the widely used radioimmunoassays and immunoenzymatic assays (enzyme-linked immunosorbent assay, chemiluminescence immunoassay, time-resolved fluorescence immunoassay).

Some genetic mutations cause complete inhibition of AFP production by the fetus. This affects the results of screening tests during pregnancy, and also leads to constantly high levels of AFP in adults, which are not linked to oncogenesis.

Key words: fetal defects; alpha-fetoprotein; isoforms; AFP-L3

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INTRODUCTION

Alpha-fetoprotein (AFP) was isolated for the first time in 1956 from the serum of human fetuses by C. Bergstrand and B. Czar during the electrophoresis of serum proteins of neonates as a fraction between albumins and alpha1-globulin [1, 2]. The oncological association of this protein with hepatocellular carcinoma was discovered first in the 1960s by G.I. Abelev and Y.S. Tatarinow [3]. Since the discovery of this relationship, over 24 000 scientific works have been devoted to the biology of AFP, its role in fetal physiology, and use in prenatal and oncological diagnostics. In the 1970s, it was considered that AFP can be highly useful in the diagnosis of neural tube defects. Consequently, the protein has been used in the triple test since the early 1990s, and in the quadruple test for the diagnosis of Down's syndrome (DS) since 1996 [4].

AFP MOLECULAR STRUCTURE AND ITS ISOFORMS

The molecular structure

AFP is a glycoprotein (contains 4.5% carbohydrates) consisting of 590 amino acids. It has a molecular mass of 69–70 kDa and a half-life of 5–6 days [4, 5]. The structure of human AFP is similar to that of other mammals and avian albumin forms [6]. Together with the genes that encode albumins, alpha-albumins, and vitamin D3-binding protein, the AFP-encoding gene forms a common family, which is located in the tandem system on chromosome 4 in locus 4q11-4q13 [5, 7, 8]. All the members of this multigene family bear similar structure and physicochemical properties. Apart from their capacity to transport different particles, they exhibit free radical-scavenging functions, esterase activity, and the ability of chemotaxis, leukocyte adhesion, and

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lipid peroxidation [8]. The proteins of the multigene family commonly have a two-dimensional alpha-helical U-form secondary structure (65–70% of the alpha-helical structure in AFP and 50% in albumins is compliant) and lack the beta-helical structure [3]. In addition, their spatial conformation is similar and comprises 3 (I–III) homologous domains, each consisting of 3 spherical subdomains conjugated by 15 regularly distributed disulphide bonds. AFP penetrates fetal and tumor cells via AFP receptors (AFP-R) and binds with the cytoplasmic proteins present in the cells. Binding of AFP with AFP-R takes place through the C-end domain (CD) in the tertiary structure. Following the binding, the AFP-R complex is internalized in the cell through endocytosis [11, 12].

Transformed AFP and growth-inhibitory peptide

Domain III of an AFP molecule contains a 34-amino-acid sequence found deep in the primary, tightly wrinkled structure. During fetal stress/shock and exposure to high concentrations of estrogen, the tertiary structure of an AFP molecule undergoes changes, resulting in „transformed AFP" and the deeply hidden 34-amino-acid sequence becomes exposed [9, 10]. This sequence has been synthesized in laboratory conditions as a growth-inhibitory peptide (GIP) and was thoroughly investigated for its biological activity. It is known that the sequence exhibits growth-inhibitory functions in fetal cells and tumor cells, in contrast to the typical primary AFP molecule [10]. GIP also prevents the local distribution and metastasis of cancers by blocking the adhesion of tumor cells to the extracellular matrix and preventing platelet aggregation. In recent years, the use of GIP to deliver chemotherapy drugs such as doxorubicin or tamoxifen into tumor cells has been extensively studied [11].

mRNA variants and isoforms of AFP

So far, numerous genetic variants of mRNA matrix have been discovered for AFP: 1.6 kb, 1.7 kb, and 2.2 kb (1 kb = 1000 base pairs). In humans, the 2.2-kb mRNA molecule is predominant (AFP matrix with a molecular weight of 69–70 kDa), while the remaining variants are found at trace levels. Interestingly, the majority of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) test sets detect AFP molecules with a molecular weight of 69–70 kDa (for 2.2 kb mRNA). Therefore, the rare isoforms are synthesized on shorter mRNA matrices and are not detected in a standard test at all. Over the last 20 years, 3 AFP isoforms — L1, L2, and L3 — have been used in scientific research, and their inclusion in clinical diagnostics has also been postulated. These isoforms were distinguished based on AFP binding with *Lens culinaris* agglutinin. AFP-L3 is predominantly found in the serum of mothers of children with DS — its transplacental passage is probably promoted relative to the other 2 isoforms. It was later proven that the use of AFP-L3 instead of AFP increases the sensitivity of the triple test [13, 14], while the use of AFP-L2 and AFP-L3 improves the detectability of open neural tube defects and abdominal wall defects [15]. AFP-L3 is the predominant isoform of AFP found in hepatocellular carcinoma based on hepatic cirrhosis and hepatitis [3].

Congenital deficiency of AFP

It is known that a gene mutation causes complete inhibition of AFP production [7, 16], which occurs at a frequency of 1 out of 105 000 neonates. This mutation occurs in exon 5 on chromosome 4 (c543 G>A) and causes the stop codon to be inserted prematurely, leading to the completion of AFP transcription. As shown by previous research, AFP is not necessary for the normal development of a fetus, because in the absence of this protein its functions are taken over by albumins and alpha-albumins. This was also proven in an animal model, in which the synthesis of AFP and albumin mRNA takes place at the same time from the early stages of pregnancy.

Hereditary persistence of AFP (HPAFP)

The last several decades of research on AFP have shown that autosomal dominant inherited gene mutation is responsible for the persistently elevated levels of AFP in adults [hereditary persistence of AFP (HPAFP)] [5, 17–19]. In such a case, constantly elevated levels of AFP, in the range of 0.009–3.564 μg/mL, are observed in the serum. From 1983 to 2010, HPAFP was recorded in 19 families. Two-point mutations on chromosome 4 were found (a-55 C>A and a-119 G>A) in the site of binding of HNF-1 (hepatocyte nuclear factor-1) to the AFP gene promoter. HNF-1 (responsible for stimulation) and NF-1 (mainly responsible for suppression) are 2 important transcription factors of the AFP gene. Genetic mutations increase the affinity of HNF-1 to the AFP promoter and cause elevated AFP transcription [5]. In addition, elevated binding of HNF-1 to the promoter results in decreased binding of NF-1 (due to the partial overlap of HNF-1- and NF-1-binding sites), which further stimulates transcription [17].

FETO-MATERNAL CIRCULATION OF AFP

AFP in fetal serum

In the first trimester of pregnancy, the production of AFP commences in the yolk sac, and concomitantly from 4 weeks in the fetal liver [4, 20], which is the predominant source of this protein. The AFP synthesis increases till 20 weeks of pregnancy, then remains constant until 32 weeks, and gradually decreases until birth [1]. Trace amounts of AFP are produced in the gastrointestinal tract and kidneys of the fetus. The protein appears

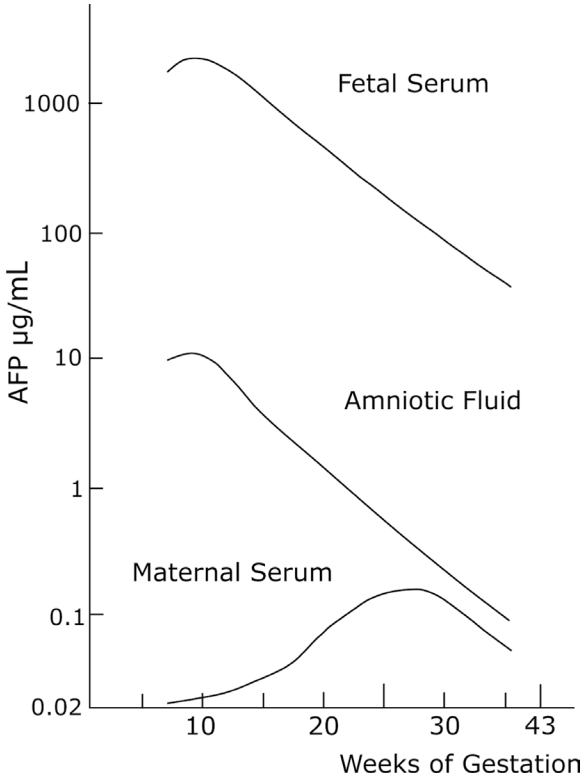

Figure 1. Alpha-fetoprotein concentration in fetal serum, maternal serum and amniotic fluid in relation to gestational age (modified from [1])

in the fetal serum 29 days postconceptional [1, 19]. It reaches the maximum concentration (approximately 3000–5000 µg/mL) at 10–13 weeks of pregnancy [21] and then gradually decreases with each week of pregnancy due to the dilution caused by the increased weight of the fetus until birth, reaching a concentration of approximately 200–300 µg/mL at 32–35 weeks and approximately 20–120 µg/mL on the day of birth [22] (Fig. 1). During the postpartum period, the gene expression undergoes changes and AFP is gradually replaced by albumins in the neonate vasculature [4, 23].

AFP in cerebrospinal fluid

AFP appears in the cerebrospinal fluid (CSF) of the fetus via filtration of the interstitial fluid from the neurons of the central nervous system and in the plasma via filtration through the blood–CSF barrier (choroid plexuses), as well as via back transport from motoneurons [24]. There are no published reference intervals for concentrations of AFP in the cerebrospinal fluid of normal fetuses. There are some short reports from investigations on AFP concentration in the CSF in aborted fetuses between 16 and 25 weeks of pregnancy. Levels of AFP are highly variable and were found to decline from 160–1220 µg/mL in 16–17 weeks of pregnancy to 60 µg/mL in 23–25 weeks of pregnancy [24, 25]. Concentrations of AFP in the CSF rapidly decrease postnatally, with a half-life of about 11 days. By the age of 6 weeks, the concentrations are close to those found in adult plasma and should be in the region of 0.014 µg/mL or less. In the cerebrospinal fluid, AFP is undetectable already after 2 months postpartum [26]. In the cases of acrania and open myelomeningocele (85% [21]), AFP is leaked from the CSF to the amniotic fluid, and from there the protein is absorbed into the maternal bloodstream [2, 28]. Such leakage does not occur in the case of closed neural tube defects, because the CSF is not washed into the amniotic fluid. Elevated AFP in the amniotic fluid may also be related to causes such as abdominal wall defects (omphalocele, gastroschisis) (Fig. 2). Thus, to confirm the relationship with neural tube defects, the concentration of acetylcholinesterase (ACHE) is determined in the amniotic fluid (typically ACHE is not present in the amniotic fluid, but only in fetal OUN) [28]. A significant finding is that elevated AFP level is not observed in the first trimester of pregnancy but only during the second trimester in the case of open myelomeningocele [31].

AFP in amniotic fluid

The maximum concentration of AFP in amniotic fluid (approximately 80 µg/mL) is reached [27] at 10 weeks of pregnancy, and the concentration gradually decreases to 0.2–3 µg/mL on the day of delivery. The AFP level in amniotic fluid decreases by about 10% per week between 14 and 20 weeks of pregnancy [21] and reaches 1% in the fetal serum at the second trimester of pregnancy [20]. In early pregnancy, AFP is introduced from the organism of the fetus to amniotic fluid through the skin, and later excreted by the kidneys with urine [20]. High fetal proteinuria indicates kidney immaturity; hence, during pregnancy, proteinuria, as well as the AFP level in the amniotic fluid, decreases [18]. AFP along with the amniotic fluid is swallowed by the fetus, and thus the protein is recirculated and then degraded in the liver. AFP penetrates the mother's bloodstream from the amniotic fluid via the amniotic sac and the decidua via a highly efficient hydrostatic gradient-based transport and, to a lesser extent, via the extracellular transport [1]. Congenital nephrotic syndrome of the Finnish type, *inherited* in an autosomal recessive manner, is an extremely rare cause of increased AFP in the amniotic fluid. This disease is caused by mutation in locus 19q13.1 in the *NPHS1* gene, responsible for coding the nephrin protein. Nephrin is an important transmembrane protein of the filtration slit in renal glomerulus, necessary for the proper functioning of the renal filtration barrier. The deficiency of nephrin results in severe proteinuria, presented in utero, typically from the second trimester of pregnancy [34].

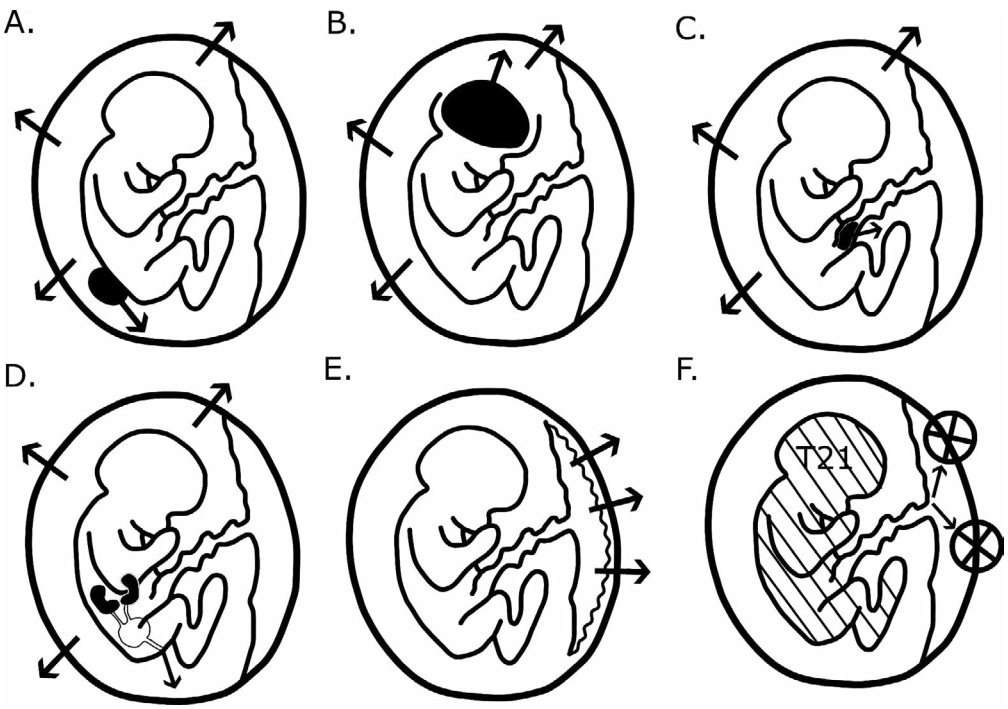

Figure 2. Fetal causes of changes in maternal serum alpha-fetoprotein (AFP) concentration; **A.** Open spina bifida: AFP increase in amniotic fluid, AFP increase in maternal serum; **B.** Acrania: the same as in A; **C.** Omphalocele: the same as in A and B; **D.** Congenital nephrotic syndrome of the Finnish type: the same as in A, B and C; **E.** Placental damage: AFP normal in amniotic fluid, AFP increase in maternal serum; **F.** Trisomy 21: AFP normal in amniotic fluid, AFP increase in placenta, AFP decrease in maternal serum

AFP in maternal serum

In the mother's serum, AFP is detected from six weeks of pregnancy [20], and its level gradually increases, reaching 0.05 µg/mL in the second trimester. The maximum concentration (approximately 1 µg/mL) is observed at 32 weeks of pregnancy, and later the concentration decreases till the day of delivery, reaching approximately 0.05–0.1 µg/mL [1] [20]. This scheme is different from the one observed in fetal serum and amniotic fluid. AFP is transported to the mother's bloodstream mainly via placental vessels with the contribution of AFP-R in the placenta and to a considerably lower extent via the amniotic sac. It has been confirmed that the placenta itself does not produce AFP [20]. The expression of receptors binding AFP placental villi is observed only in the second and third trimester, and not in the first [23], and until that time, AFP is transported through the amniotic fluid and amniotic sac. Transplacental transport is challenged by 4 barriers: syncytiotrophoblast immersed in the maternal blood in the intervillous space, trophoblast basement membrane, endothelial basement membrane, and fetal vascular endothelium. The transplacental transport is predominantly unidirectional — from fetus to mother — and takes place in 2 ways: in the first route, the protein leaves the fetal vessel lumen through the core of the placental villi and is transitioned through discontinuities/cavities within the syncytiotrophoblast; in the second route, the protein is transported through the recovery vessels passing through the decidua. The transport is facilitated by the hydrostatic gradient between the high pressure in fetal vessels and the low pressure in the intervillous space [8]. In the case of a genetically and anatomically healthy fetus, increased fetus–mother transport of AFP occurs even if the placental barrier is damaged at the level of placental villi [29]. On the other hand, defective placental transport of AFP is observed in the case of DS, and thus the low level of AFP in the maternal serum, despite normal fetal production. This theory is further confirmed by the histopathological determination of high AFP levels in the placenta [20, 30] (Tab. 1).

REFERENCE VALUES AND LABORATORY DETERMINATION METHODS

Based on the long-term screening programs used to detect neural tube defects and fetuses with DS, it was determined that the AFP norms in maternal serum range from 0.5 to 2.5 MoM in the first and second trimester [32, 33]. Until the 1970s, AFP detection was widely carried out by immunoelectrophoresis, which is a method focusing on qualitative determination and does not produce accurate results. In the 1970s, RIA methods were introduced, which utilized anti-AFP antibodies labeled with I125 and enabled AFP detection in the range of 0.002–0.005 µg/mL. This led to the inclusion of AFP analysis on a wide scale in screening

Table 1. Normal ranges of AFP in corelation to compartment, gestational age and period of life (wks = weeks of gestation)

AFP (compartment)		Physiological ranges
Fetal serum	10–13 wks	3000–5000 µg/mL [21]
	32–35 wks	200–300 µg/mL [22]
	40 wks	20–120 µg/mL [22]
Cerebrospinal fluid	16–17 wks	160–1220 µg/mL [24, 25]
	23–25 wks	60 µg/mL [24, 25]
Amniotic fluid	10 wks	80 µg/mL [27]
	40 wks	0.2–3 µg/mL [27]
Maternal serum	32 wks	ca 1 µg/mL [20]
	40 wks	0.05–0.1 µg/mL [20]
HPAFP mutation		0.009–3.564 µg/mL [5, 17–19]

AFP — alpha-fetoprotein; HPAFP — hereditary persistence of AFP

tests used for the diagnostics of neural tube defects [2]. Subsequently, ELISA tests were introduced, which are based on the use of anti-AFP antibodies labeled with the enzyme responsible for the transformation of a substrate into a colored product. The 1990s were marked by the development of immunoenzymatic techniques. Then, chemiluminescence immunoassay was introduced, involving the use of anti-AFP antibodies with a chemiluminescent marker. In this technique, following the bonding between 2 antibodies and the addition of a substrate, a light reaction is produced, which is measured with a chemiluminometer. The latest method of AFP determination is time-resolved fluorescence immunoassay, which is based on the use of antibodies labeled with lanthanide chelates—typically europium isotopes, because they are characterized by long radioactive decay times, considerably longer than typical fluorescing compounds. In this technique, following the binding of antibodies with AFP and addition of a booster solution, lanthanide is released from the bond with the antibody and chelate is bound with the fluorescing compound, responsible for transferring the triggering wave onto the lanthanide; subsequently, the triggering wave is emitted and measured (Tab. 2).

CONCLUSIONS

- In numerous gestational complications, pathologically high levels of AFP are observed in the serum of a pregnant woman, which is not related to elevated fetal production, but the increased fetal–maternal leak due to placental injury.
- AFP determination can be useful for the diagnosis of neural tube defects only from the second trimester and only in the case of "open" defects, because in such case elevated release of AFP with CSF to the amniotic fluid occurs.

Table 2. Analitycal methods and their accuracy (detection limits) in AFP measurements [35–38]

Method	Accuracy
RIA	From 5×10^{-4} µg/mL
ELISA	From 6×10^{-7} µg/mL
CLIA	From 6×10^{-5} µg/mL
TRFIA	From 1.21×10^{-4} µg/mL

CLIA — chemiluminescence immunoassay; ELISA — enzyme-linked immunosorbent assay; RIA — radioimmunoassay; TRFIA — time-resolved fluorescence immunoassay

- In the case of "closed" neural tube defects, increased AFP level is not observed in the mother's serum, because such defects do not feature AFP leakage with CSF to the amniotic fluid.
- Defective placental transport of AFP is observed in DS; thus, the level of AFP is low in the maternal serum, despite normal fetal production.
- Congenital nephrotic syndrome of the Finnish type is a rare cause of increased AFP in the amniotic fluid and maternal serum, characterized by severe fetal proteinuria, starting from the second trimester of pregnancy.
- Rare genetic mutations cause complete inhibition of AFP production by the fetus. This leads to erroneously negative results in screening tests during pregnancy, and constantly high levels of AFP in adults, which are not linked to oncogenesis, thus resulting in erroneously positive tumor diagnosis.

Conflict of interest

All authors declare no conflict of interest.

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