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Invited critical review

# Afamin — A pleiotropic glycoprotein involved in various disease states

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## ARTICLE INFO

## ABSTRACT

The human glycoprotein afamin was discovered as the fourth member of the albumin gene family. Despite intense research over the last 20 years, our knowledge of afamin's physiological or pathophysiological functions is still very limited. Circulating afamin is primarily of hepatic origin and abundant concentrations are found in plasma, cerebrospinal, ovarian follicular and seminal fluids. In vitro binding studies revealed specific binding properties for vitamin E. A previously performed analytical characterization and clinical evaluation study of an enzyme-linked immunosorbent assay for quantitative measurement of afamin in human plasma demonstrated that the afamin assay meets the quality specifications for laboratory medicine. Comparative proteomics has identified afamin as a potential biomarker for ovarian cancer and these findings were confirmed by quantitative immunoassay of afamin and validated in independent cohorts of patients with ovarian cancer. Afamin has also been investigated in other types of carcinoma. Most of these studies await further evaluation with validated quantitative afamin assays and require validation in larger patient cohorts. Transgenic mice overexpressing the human afamin gene revealed increased body weight and increased blood concentrations of lipids and glucose. These transgenic mouse data were in line with three large human population-based studies showing that afamin is strongly associated with the prevalence and development of the metabolic syndrome. This review summarizes and discusses the molecular, biochemical and analytical characterization of afamin as well as possible clinical applications of afamin measurement.

## Contents

# 1. Introduction

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Human afamin is a glycoprotein that is present in biological fluids such as plasma, and cerebrospinal, ovarian follicular and seminal fluids. Afamin was discovered in 1994 by Lichtenstein et al. as the fourth member of the albumin gene family [1]. Despite intensive research in the last

20 years, our knowledge of afamin's physiological or pathophysiological functions is still very limited. Most published work has so far focused on the molecular and biochemical characterization of afamin and on discovery studies using proteomics and epidemiological approaches to search for (patho)-physiological functions. Suitable cell lines expressing human afamin have not yet been reported and the precise molecular structure is also unknown, which explains our limited knowledge of afamin functions, particularly at the mechanistic molecular level.

# 2. Biology of afamin

## 2.1. First discoveries and characterizations

Human afamin (AFM) was discovered by sequence analysis and cloning in 1994 as the fourth member of the human albumin gene family, which includes albumin (ALB), α-fetoprotein (AFP) and vitamin D-binding protein (DBP) [1]. Afamin was previously described independently by three other research groups as α-albumin in the rat [2], as α-1T-glycoprotein in humans [3], and was first reported as a tryptophan-poor α₁-glycoprotein 50 years ago in Clinica Chimica Acta [4]. Thus, the year 2014 marked not only the 20th anniversary of its first molecular characterization, but also the 50th anniversary of its initial protein-chemical discovery and description in 1964.

All four genes of the albumin gene family map to the chromosomal region 4q11–q22 and are tandemly linked in the sub-centromeric region 5′ALB–5′AFP–5′AFM–5′DBP3′-centromere, and hence are transcribed in the same, centromere-bound, direction [1,5]. The linear chromosomal arrangement of the four genes and the structural differences between them are congruent with the following evolutionary divergence of the gene family: starting with the first duplication of an ancestral progenitor gene, a single evolutionary line led to the contemporary DBP, leaving ALB/AFP/AFM on the other line of descent. The second duplication occurred in this ALB lineage, giving rise to ALB and the AFP/AFM progenitor, while the third one gave rise to the AFP–AFM pair. Recently, a new, fifth member of the albumin gene family was discovered, located adjacent to the 3′ end of the AFM gene but structurally related to AFP, which is why it was named α-fetoprotein-related gene (ARG) [6]. The ARG gene is expressed perinatally at very low levels in the liver of mice, rats, dogs and horses, but is considered an inactive pseudogene in humans and other primates.

The nucleotide sequence of the human afamin gene spanning 24.454 bp was first reported by Nishio et al. revealing a gene structure of 15 exons separated by 14 introns [7]. Based on structural similarity, α-albumin appears to be most closely related to α-fetoprotein. The complete structure of this family of four tandemly linked genes provides a well-characterized 200 kb locus in the 4q sub-centromeric region of the human genome.

Human afamin is a glycoprotein with an apparent molecular weight of 87 kDa and 55% amino acid sequence similarity (34% identity) to albumin [1] but, in contrast to albumin, is highly and in a complex manner glycosylated [3,8]. The afamin sequence completely lacks tryptophan [1], in line with its initial description of a tryptophan-poor glycoprotein [4]. The polypeptide chain is composed of a 21-amino acid leader peptide, followed by 578 amino acids of the mature protein. Afamin has five predicted N-glycosylation sites, and treatment with N-glycanase reduces the apparent molecular mass of afamin to 65 kDa [1,3]. Like other members of the albumin multigene family, afamin consists of three structural domains containing 17 Cys–Cys disulfide bridges [1].

At the same time as human afamin was discovered, the rat ortholog of afamin was described by Bélanger et al. [2]. In the rat, liver expression of afamin starts at birth and continues in adult animals, in contrast to α-fetoprotein which is heavily expressed in yolk sac, fetal and newborn liver, but not in the adult liver. Therefore, in rats afamin can be considered the adult form of α-fetoprotein. In extrahepatic rat tissues such as kidney and brain, afamin is not expressed. Rat afamin reveals six predicted N-glycosylation sites [2]. Allard et al. estimated the rat serum concentration of afamin at approximately 20 mg/L [9].

## 2.2. Gene regulation of afamin

The tight linkage between the members of the albumin gene family and their liver-specific expression has prompted the suggestion that these genes share common regulatory elements. The α-fetoprotein enhancer region that activates α-fetoprotein and albumin in fetal liver development does not, however, affect the expression of all gene family members, including afamin, later in hepatic development [10]. Two hepatocyte nuclear factor 1 (HNF1)-binding sites binding HNF1α and HNF1β have been identified in the afamin promoter. Expression studies in mice suggest that the different responsiveness of albumin gene family members is crucial for their liver expression [11]. More recently, a microarray study showed increased expression of transcription factor islet-1 levels (which is associated with increased β-cell function) to increase afamin expression 35-fold [12].

In summary, our understanding of afamin's gene regulation is very limited and needs to be further explored using suitable cell lines and/or animal models.

## 2.3. Biochemical and functional characterization

Human plasma afamin has been shown to be a specific binding protein for vitamin E [13]. Radio ligand-binding assay followed by Scatchard and Hill analyses demonstrated in vitro afamin's specific binding affinity for both α-tocopherol and γ-tocopherol, two of the most important forms of vitamin E. Maximum 18 binding sites for vitamin E per molecule afamin was estimated. The binding dissociation constant was determined to be $18.0 \pm 7.1$ μM, indicating that afamin might play a role as vitamin E carrier in plasma and other body fluids under physiological conditions. Furthermore, a Hill coefficient of 1.8 was obtained indicating a slight positive cooperativity that can best be explained by the fact that incoming, hydrophobic α-tocopherol molecules increase the hydrophobicity of the protein–ligand complex and thus make this complex more accessible to forthcoming ligands. Due to the large binding capacity of afamin for vitamin E, it might take over the role of vitamin E transport in body fluids under conditions in which the lipoprotein system is not sufficient for vitamin E transport. Specific binding of vitamin E was also confirmed by means of surface plasmon resonance technology with afamin immobilized on carboxymethyl dextran surface chips [13].

In view of the described experimental evidence, homology modeling and docking calculations were performed on the predicted tertiary structure and demonstrated coincidence between calculated and in vitro results (Fig. 1) [8,13].

Several studies reported quantitative and qualitative analyses of afamin's substantial glycan decorations. First reports date back to the initial discovery of afamin by Haupt and Heide in 1964, who reported a carbohydrate content of 13% [4]. This value is in remarkable agreement with more recent reports by Jerkovic et al., who found 15% carbohydrates by quantitative high performance liquid chromatography [8], but stands in contrast to data from Lichenstein et al., who estimated 24% carbohydrates based on less accurate comparisons of electrophoretic separation between fully glycosylated and enzymatically deglycosylated afamin [1].

Two reports on compositional glycan analysis of afamin demonstrated that >90% of glycans are sialylated biantennary complex structures bound to five N-glycosylation sites [3,8]. One of these amino acid residues, Asn362, possesses a rare consensus sequence for N-glycan of Asn-X-Cys. Afamin also contains five potential O-linked glycosylation sites; there is, however, no evidence of O-glycosylation [8]. Immunoblot analyses of normal human plasma using afamin-specific antibodies after 2-dimensional gel electrophoresis revealed substantial molecular heterogeneity of afamin believed to be due to different glycosylation
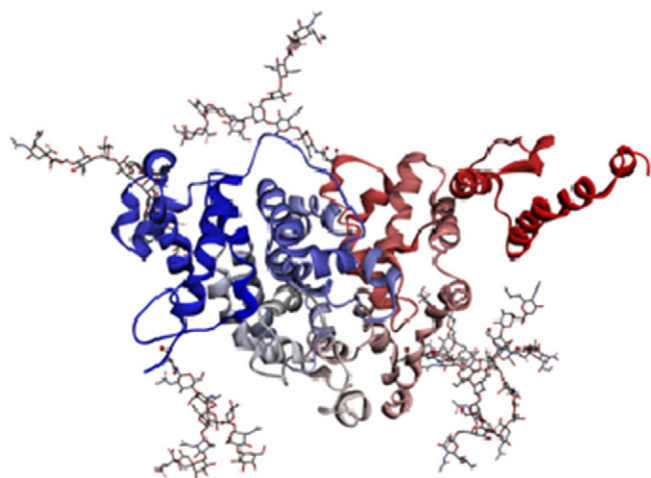
**Fig. 1.** Ribbon presentation of homology model of human afamin. The figure was generated with Accelrys Discovery Studio Visualizer 3.5 from coordinates of a homology model based on the albumin crystal structure and energy-minimized glycans as published by Jerkovic et al. [8]. The ribbon presentation of the peptide chain is colored from N-terminal (blue) to C-terminal (red) while the putative glycans are shown as ball-and-stick models.

patterns [8]. So far, there is no experimental evidence for any functional role of the substantial glycan content of afamin.

To investigate a possible association between afamin and the plasma lipoprotein system (where most plasma vitamin E is transported [14]), plasma distribution of afamin was investigated by size-exclusion chromatography. Afamin eluted between apolipoprotein AI-containing fractions (representing high-density lipoproteins (HDL)) and lipid-free protein fractions [8]. Incomplete separation and some overlap with HDL and lipid-free fractions suggest a partial association between afamin and HDL (sub)fractions. To quantify this potentially lipoprotein-associated fraction of afamin, the afamin concentration in human plasma samples was measured prior to and post lipoprotein precipitation. In line with the observation made with size-exclusion chromatography, 13% of afamin was found to be lipoprotein-associated. Using this method more than 97% of vitamin E was seen to be lipoprotein-associated, thus confirming that vitamin E is almost completely transported via the lipoprotein system in plasma [14]. These data also agree with results from our Surface-Plasmon-Resonance experiments that showed specific vitamin E-binding of afamin, but no binding with human serum albumin [13], in contrast to a recent report demonstrating in vitro vitamin E-binding by albumin [15].

The observed almost exclusive transport of vitamin E via the lipoprotein system is also in line with association studies of afamin and vitamin E concentrations in various body fluids. Afamin significantly correlates with vitamin E in ovarian follicular and cerebrospinal fluids, but not in plasma [8]. Afamin's association with vitamin E in ovarian follicular fluid was directly demonstrated by gel filtration chromatography and immunoprecipitation, which complements the in vitro findings for purified native and recombinant afamin and suggests vitamin E carrier functions for afamin in body fluids with lipoprotein species not suitable for vitamin E transport.

## 3. Afamin expression and occurrence in human body fluids

In addition to the abundant occurrence of afamin in human plasma, comparatively high concentrations of afamin have also been described in other human body fluids such as cerebrospinal, ovarian follicular, and seminal fluids [3,8,16–19]. The brain, kidney, testes and ovaries have been found as additional afamin-expressing tissues (www.proteinatlas.org). It is, however, completely unclear whether afamin

expressed in these tissues contributes to circulating plasma afamin and what physiological roles afamin plays in these organs.

Kratzer et al. discovered afamin expression in cerebrovascular endothelial cells and demonstrated that afamin facilitates vitamin E transport/transfer across the blood–brain barrier in a suitable cell culture model [20], suggesting a role of afamin in the regulation of vitamin E uptake and transport at the blood–brain barrier.

Furthermore, in an in vitro chicken neuronal culture model afamin was shown to possess neuroprotective properties when these cells were challenged by apoptotic treatment [21]. Future studies need to investigate whether these findings in a model cell system have any potential therapeutic significance in animal models or humans.

Finally, afamin expression was previously shown for the first time in differentiated osteoclasts derived from mouse bone marrow with potential impact on bone resorption via G-coupled receptor and $Ca^{2+}$/calmodulin-dependent protein kinase signaling pathways [22,23]. The authors discussed afamin as a potential chemokine involved in osteoblast metabolism and bone formation. Currently there is no information regarding similar roles of afamin in human bone metabolism.

## 4. Analytical characterization and clinical evaluation of a human afamin enzyme-linked immunosorbent assay (ELISA)

We previously performed a thorough analytical characterization and clinical evaluation of a custom-made ELISA (MicroCoat Biotechnologie, Bernried, Germany) for quantitative measurement of afamin in human plasma [16]. This assay is of the double-antibody sandwich type and consists of an affinity-purified polyclonal rabbit anti-human afamin antibody for analyte capture and a peroxidase-conjugated mouse monoclonal antibody for detection. A 7 mg/L detection limit was reported, using a diluted sample from a healthy individual. This evaluation study revealed a within-run and total CV < 10% for the afamin assay. Furthermore, the afamin assay was linear across the tested measurement range (18–77 mg/L) [16]. Studies on the in vitro stability of afamin indicated that afamin is stable for 24 h at room temperature, for 48 h at 4 °C, and for at least one year at −20 °C and −80 °C [16]. The afamin assay is thus well suited for use in routine laboratory setting and the analyte stability of afamin allows for convenient specimen shipment and storage.

The reference value study revealed that afamin concentrations do not differ between males and females and are not associated with age or renal function in healthy blood donors (Fig. 2). Thus, the age- and sex-independent reference interval was 45–99 mg/L (median 68 mg/L, range 33–113 mg/L) using a non-parametric percentile method (95%, double-sided) [16].

We also studied the components of biological variation of afamin in healthy individuals at one-week intervals for six weeks and found a reference change value of 24% [16]. The reference change value takes into account the within-subject biological variation as well as the analytical variation and indicates the difference required for two serial measurements of afamin to be significantly different at p < 0.05.

No significant differences in afamin concentrations between serum and plasma samples or between fasting and non-fasting state were seen, indicating that serum and plasma samples are both suitable for afamin measurement and that afamin concentrations can be correctly determined independently of a patient's fasting state [16].

Evaluation of the diurnal profile of afamin and the potential influence of menstrual cycle phase on afamin in healthy females revealed neither a circadian variation nor a relevant influence of menstrual phase on afamin concentrations [16]. Together with the data retrieved from the biological variation study, these findings underline the low variation of afamin over time, demonstrating good suitability of afamin for serial measurements.

As part of the clinical evaluation of the afamin assay plasma concentrations of afamin were investigated in patients with various diseases including patients with heart failure, pneumonia, chronic obstructive
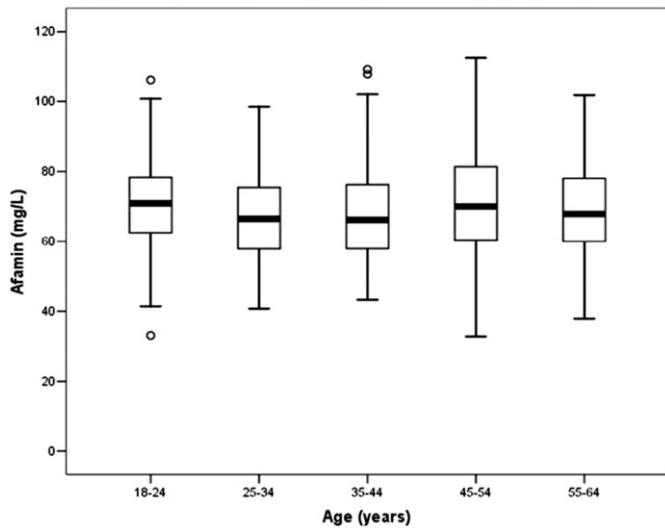
**Fig. 2.** Box-and-whisker plots showing afamin plasma concentrations in 528 healthy blood donors across different age groups. Median afamin plasma concentrations according to age group: 18–24 years (n = 94), 71 mg/L (range 33–106 mg/L); 25–34 years (n = 131), 66 mg/L (range 40–98 mg/L); 35–44 years (n = 128), 66 mg/L (range 43–109 mg/L); 45–54 years (n = 127), 70 mg/L (range 33–113 mg/L); and 55–64 years (n = 48), 68 mg/L (range 38–102 mg/L). No significant differences in afamin plasma concentrations were observed across these age groups (Kruskal–Wallis test, p = 0.104).

pulmonary disease, renal disease and sepsis and compared with those of healthy controls [16]. In this explorative analysis afamin plasma concentrations were modestly decreased in patients with heart failure. Patients with pneumonia or sepsis exhibited markedly decreased afamin plasma concentrations. In contrast, patients with chronic renal disease or chronic obstructive pulmonary disease showed no difference in afamin plasma concentrations as compared to healthy individuals. In this context it is noteworthy that in a convenient sample of hospitalized patients with a variety of diseases afamin showed a rather strong inverse association with the inflammatory biomarkers C-reactive protein (CRP) and interleukin-6 [16]. These results revealed novel insights into afamin as a negative acute phase protein and should encourage further studies.

## 5. Clinical applications of afamin measurement

Several studies in search of (patho)-physiological functions of afamin have been conducted, mostly using proteomics and/or epidemiologic approaches with a population-based or case–control study design. Many of these studies were of a discovery nature only with small sample sizes and were not evaluated in larger cohorts.

Comparative proteomics has previously identified afamin as a potential biomarker for ovarian cancer [18]. These findings were confirmed with immunoblotting and a quantitative immunoassay for afamin. Patients with ovarian cancer displayed significantly decreased plasma concentrations of afamin by comparison to healthy controls. These results were later validated in an independent larger study of patients with ovarian cancer [17] and, very recently, extended by showing significant associations between afamin plasma concentrations and clinical outcomes (response to therapy and survival rates) [24]. In contrast, afamin concentrations were not found to be decreased in benign gynecological conditions including endometriosis [17,25].

Circulating afamin concentrations have also been investigated in various other types of carcinoma including gastric, bladder, colorectal, cervix, breast, and thyroid cancer. Most of these studies, however, await evaluation by quantitative analyses using appropriate methods in sufficiently large study groups [26–33]. Of these many studies, two may highlight the importance of proper validation studies after a potential tumor marker candidate has been discovered [29,33]. Penno et al.

described in a mouse model of gastric cancer eight proteins (including afamin) as being differently expressed compared to healthy wild-type mice. Very recently, the same group aimed to validate their data from the mouse model in a small group of patients with diagnosed gastric cancer (n = 37) in comparison with an even smaller number of healthy control subjects (n = 7), using a commercially available afamin ELISA from Uscn Life Sciences (Wuhan, China). The authors reported lower afamin concentrations in gastric cancer patients than in healthy controls [29]. Interestingly, they found median afamin plasma concentrations in their healthy controls to be approximately 3-fold higher than the reference values previously reported in 528 healthy blood donors [16]. The reasons for the lack of agreement between the two ELISA methods are most probably due to different standards and/or different antibodies, and also different reagents and buffers. Therefore, it is important to be aware that the results reported in published studies obtained with different methods are not directly comparable. Understanding differences between these assays is critical, as they obviously vary quite substantially.

In view of the reported nervous tissue expression and putative neuroprotective properties of afamin [20,21], several recent (mostly discovery) studies revealed possible marker properties of afamin for various neurological pathologies including Alzheimer's disease and multiple sclerosis [34–36]. It will be very interesting to see whether these findings can be confirmed in large human epidemiological studies.

During uncomplicated pregnancy afamin increases linearly almost two-fold and drops to pre-pregnant values immediately after delivery [37]. It is therefore 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 of hepatic synthesis of lipids and lipoproteins leading to physiological hyperlipidemia during gestation [38].

In a pilot study conducted in patients diagnosed with pregnancy complications, blood drawn at the first trimester screening from women with preeclampsia revealed significantly higher median afamin concentrations than did blood from women with uncomplicated pregnancy [37]. These findings suggest a potential role of afamin as a predictive marker for pregnancy-related disorders. However, further prospectively planned and adequately powered studies are needed to confirm the potential role of afamin as a marker for pregnancy complications.

Very recently, the first large, population-based epidemiological study in search of (patho)-physiological functions of afamin was performed [19]. The authors focused their analyses on selected phenotypes associated with afamin after having previously investigated transgenic mice overexpressing the human afamin gene revealing increased body weight and serum concentrations of lipids and glucose. To validate these data from a pilot animal study in a large human epidemiological study, a random-effects meta-analysis using age- and sex-adjusted baseline and follow-up investigation was applied in three large population-based cohorts from Northern Italy, Austria and Southern Germany. Mean afamin concentrations were $63 \pm 15$, $66 \pm 14$, and $71 \pm 17$ mg/L in the Bruneck, SAPHIR and KORA F4 studies, respectively. Per 10 mg/L increment in afamin measured at baseline, the number of metabolic syndrome components increased by 19% (incidence rate ratio (IRR) = 1.19 (95% CI 1.16–1.21), $p < 0.0001$). With the same afamin increment as used at baseline an 8% gain in metabolic syndrome components between baseline and follow-up was observed (IRR = 1.08 (95% CI 1.06–1.10), $p < 0.0001$). Afamin concentrations at baseline were highly significantly related to all individual metabolic syndrome components at baseline and follow-up.

In contrast to a previous small study in hospitalized patients with various diseases where a rather strong inverse association between afamin and CRP was reported ($r_s -0.463$, $p < 0.001$) [16], a weak but significant positive association between afamin and CRP was found in the three large population-based studies ($r_s +0.108$, $p < 0.01$ in

Bruneck, $r_s$ + 0.222, p < 0.001 in SAPHIR, and $r_s$ + 0.213, p < 0.001 in KORA F4) [19]. A possible explanation for this discrepancy might be a different grade of inflammation present in acutely diseased patients (e.g., chronic obstructive pulmonary disease exacerbation and pneumonia) needing hospitalization versus low-grade chronic inflammation in population-based cohorts. This is, however, mainly speculative and further studies are necessary to clarify this issue.

Taken together, this study in transgenic mice and more than 5000 participants in epidemiological studies showed that afamin is strongly associated with the prevalence and development of metabolic syndrome and all its components. In line with these findings, elevated afamin concentrations were recently demonstrated in patients with polycystic ovary syndrome, which is associated with insulin resistance, thus confirming afamin's role in a comparable metabolic disorder also in patients with polycystic ovary syndrome [39,40].

Further large epidemiologic and functional studies must be performed in order to shed more light on the role of afamin in metabolic syndrome-related pathologies such as diabetes, obesity and subsequent clinical endpoints of cardiovascular diseases (Fig. 3).

## 6. Conclusions and future perspectives

Afamin is a human plasma vitamin E-binding glycoprotein primarily expressed in the liver and secreted into the bloodstream. Our current knowledge of afamin's possible physiological or pathophysiological functions is still very limited and is summarized in Fig. 3.

Previously, a comprehensive analytical characterization and clinical evaluation of an enzyme-linked immunosorbent assay for quantitative measurement of afamin in human plasma were performed and demonstrated that this afamin assay meets the quality specifications for laboratory medicine. Afamin shows good in vitro stability, which is important as a preanalytical issue. The biological variation of afamin is rather low, implying good suitability of afamin for serial measurements. The results of the clinical evaluation in hospitalized patients with various diseases revealed novel insights into afamin as a negative acute-phase protein. In contrast, we found a positive association between afamin and CRP in three large population-based cohorts. Hence, additional studies are necessary to clarify this issue.

Although several studies have identified afamin in different diseases by proteomic approaches, as mentioned above, thorough validation with properly standardized quantitative afamin assays is missing for many of these pilot studies. Obviously, in the absence of proper validation, afamin concentrations might be measured incorrectly.

In this context, a recent study of pooled plasma samples using multiplexed mass spectrometry-based technology was able to simultaneously measure 142 plasma proteins including afamin [41]. Even though this seems to be a promising new technology, the results should be interpreted with great care. The authors of this study reported almost 3-fold increased afamin values compared to those obtained in our laboratory and by others [3,16]. Since this methodology is calibrated with selected peptides, the specificity of the assay might suffer, particularly when an attempt is made to quantify several proteins from the same gene family.

A newer version of the afamin ELISA evaluated in our laboratory [16] is now commercially available from BioVendor, Brno, Czech Republic. This assay differs from our previously used system by using two different monoclonal antibodies with defined epitope recognition and a primary protein standard consisting of recombinantly expressed human afamin. This modification allows the afamin assay components to be produced and maintained with improved lot-to-lot stability and
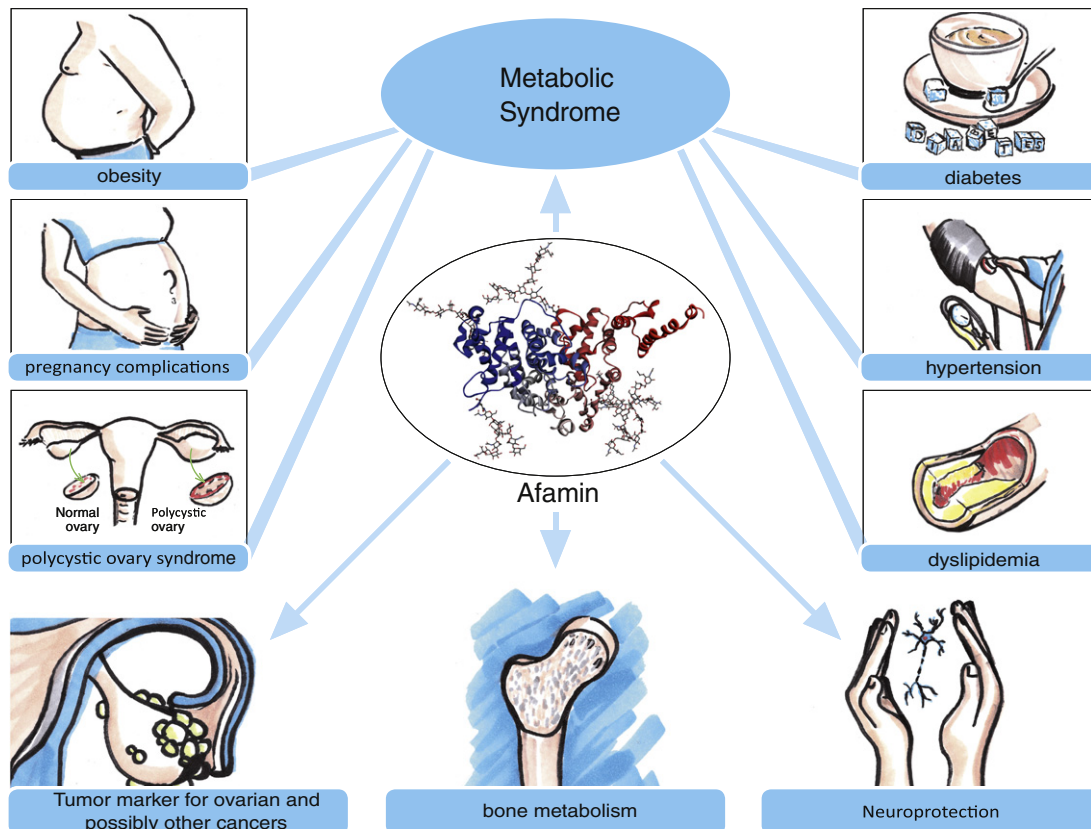


**Fig. 3.** Summary of putative (patho)-physiological functions of afamin. Blood concentrations of afamin have been found to be associated with a variety of disease phenotypes including metabolic syndrome and related pathologies such as obesity, pregnancy complications and polycystic ovary syndrome, type-2 diabetes, hypertension and dyslipidemia. In addition, afamin was described as a tumor marker for ovarian cancer and possibly other cancer types and may play a regulatory role in bone metabolism and signaling pathways. Finally, afamin exerts neuroprotective properties in vitro, facilitates vitamin E transport across the blood–brain barrier and is expressed in nervous tissue. A role of afamin as marker for neurological disease is currently discussed.

reproducibility. To our knowledge, there is currently one other afamin ELISA assay from Uscn Life Science Inc., Wuhan China commercially available. However, there is no published data on an analytical evaluation of this afamin ELISA assay.

Future research efforts regarding this pleiotropic glycoprotein will range from extended epidemiological studies including genetic association studies, other phenotypes related to metabolic syndrome and clinical endpoints to functional studies using appropriate liver cell culture and genetically modified animal models. Finally, structure–function studies of purified human afamin will unravel further physiological ligands and binding partners, which will eventually lead us to discover hitherto unknown (patho)-physiological functions of afamin.

## Financial & competing interest disclosure

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