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MALE REPRODUCTION: ORIGINAL ARTICLE

Seminal Plasma and Serum Afamin Levels Are Associated with Infertility in Men with Oligoasthenoteratozoospermia

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Abstract

The plasma glycoprotein afamin has been previously identified as an alternative carrier protein for vitamin E in extravascular fluids such as plasma and cerebrospinal, ovarian follicular, and seminal fluids. However, to date, no study has established a relationship between afamin levels and infertility in women or men. The purposes of our study were (i) to assess the level of afamin in serum and seminal fluids in infertile men compared to healthy controls and (ii) to study the association between polymorphisms in afamin genes and male infertility. This observational, prospective study evaluated the afamin levels in serum and seminal fluids from infertile men ($n = 39$) and compared them to those in healthy controls ($n = 30$). We studied the association between single-nucleotide polymorphisms (SNPs) in the 5`-untranslated region (5`-UTR) of the afamin gene and infertility and analyzed a total of 1000 base pairs from the untranslated region of the afamin gene. Subjects with low sperm motility and low sperm concentration had higher median seminal afamin (18.9 ± 2.9 ng/mg of proteins) and serum afamin concentrations (24.1 ± 4.0 ng/mg of proteins) than subjects with normal sperm parameters (10.6 ± 1.4 ng/mg of proteins) ($p < 0.02$) (15.6 ± 1.4 ng/mg of proteins) ($p < 0.002$). A total of five different polymorphisms were found, including one deletion and four single-nucleotide polymorphisms (SNPs). A new transversion (A/T) (position 4:73481093) was identified in an oligoasthenoteratozoospermic patient and was associated with high levels of afamin in plasma and seminal fluids. The prevalence of this variant in our study in the case homozygous for TT is 0.985 (98.5%), and in the case heterozygous for TA is 0.015 (1.5%). Our results suggest that genetic variations in afamin might be associated with male infertility. These findings could significantly enhance our understanding of the molecular genetic causes of infertility.

Keywords Afamin · Male infertility · Oligoasthenoteratozoospermia · Seminal plasma · Polymorphisms genes

This article was updated to correct errors in Table 1 and References 7 and 27.

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Introduction

The glycoprotein afamin (AFM) was discovered in 1994 by two independent research groups, in humans [1] and in rats [2], as the fourth member of the albumin gene family. The afamin gene is located on chromosome 4q11–q13 in humans [1]. Afamin is a human plasma glycoprotein of 87 kDa with 15% carbohydrate content and 55% amino acid sequence similarity to albumin [1]. Afamin is primarily expressed in the liver and is secreted into blood with subsequent distribution to the various extravascular fluids [1].

Afamin has been previously identified and described as an alternative carrier protein for vitamin E [3, 4] in extravascular fluids, such as follicular fluid, which is known for its reduced lipoprotein content [5], suggesting a role for afamin in female fertility, i.e., follicle maturation. High levels of afamin have also been described in other body fluids, such as cerebrospinal fluid

and seminal plasma [6, 7]. Afamin concentrations were found to correlate with those of vitamin E in follicular and cerebrospinal fluids but not in plasma [3]. This may indicate a dissociation of afamin from its vitamin E carrier function in plasma, in contrast to its physiological carrier function in follicular and cerebrospinal fluids. It is, however, unknown whether the afamin expressed in these tissues contributes to circulating plasma afamin and what physiological roles afamin plays in these organs [7].

Afamin may play a role in oxidative stress–related antiapoptotic cellular processes. Heiser et al. isolated cortical chicken neurons either maintained under apoptosis-inducing low-serum conditions or exposed to oxidative stress by the addition of H_2O_2 or beta-amyloid peptide. Afamin and vitamin E synergistically enhance the survival of cortical neurons under apoptotic conditions [6].

Lambrinoudaki et al. [8] demonstrated that several markers of oxidative stress are elevated in the serum of women with endometriosis, suggesting a systemic phenomenon. Afamin levels were altered significantly in the peritoneal fluid of women with endometriosis compared with disease-free controls and correlated with vitamin E levels, a finding that confirms the link between the disease and localized oxidative stress [9]. These authors postulated that the higher afamin content in stage I and II endometriosis may be an indication of a more acute stage of disease and suggest an active recruitment of antioxidants, including vitamin E, to this local inflammatory environment.

Serum afamin concentrations are elevated in the first trimester among women who develop preeclampsia compared to controls [10]. Moreover, afamin may serve as an early biomarker for pathological glucose metabolism during pregnancy [11]. These results suggest a potential role of afamin as a predictive marker for pregnancy-related disorders.

Several studies have revealed a possible role for afamin as a marker for various neurological pathologies, including Alzheimer's disease and multiple sclerosis [12–14].

Comparative proteomics has also identified afamin as a potential biomarker for ovarian cancer [15]. These findings were confirmed with immunoblotting and a quantitative immunoassay for afamin. Patients with ovarian cancer displayed significantly decreased plasma concentrations of afamin compared to healthy controls. These results were later validated in an independent larger study of patients with ovarian cancer [16] and extended by showing significant associations between afamin plasma concentrations and clinical outcomes (response to therapy and survival rates) [17].

Circulating afamin concentrations have also been investigated in other types of carcinoma, including bladder, colorectal, gastric, cervix, breast, and thyroid cancer [18–23], and have also been associated with the prevalence and development of metabolic syndrome [24], type 2 diabetes [25], and diabetic nephropathy [26].

The role of afamin in male and female fertility was discovered in an afamin knockout mouse model. These mice were completely infertile even at the chimeric stage of afamin gene deletion. Male mice showed disrupted testis histology, impaired spermatogenesis, and decreased testis organ size. The central importance of afamin in fertility was further supported by the complete restoration of fertility and testes histology after exogenous application of recombinant expressed mouse afamin in previously infertile chimeric animals. These results from a gene-deleted mouse model indicate a central role of afamin in fertility, possibly due to its vitamin E–binding properties [27].

More recently, afamin was found to be associated with premature ovarian insufficiency, suggesting that this protein may participate in the pathogenesis of premature ovarian failure [28].

Despite this extensive research, our knowledge of afamin's functions is still extremely limited, especially at the molecular level [7].

Moreover, to date, no study has investigated the relationship r between the levels of afamin protein, polymorphism, or expression of afamin genes and male infertility. The precise role of afamin in human reproduction is virtually unknown. Therefore, the purposes of our study were (i) to assess the level of afamin in serum and seminal fluids of infertile men compared to healthy controls and (ii) to study the association between polymorphisms in the afamin gene and male infertility.

Material and Methods

Study Population

This observational, prospective study evaluates the afamin levels in the serum and seminal fluids of infertile men and compares them to those of healthy controls.

A total of 69 men aged 18–55 years were consecutively enrolled in the study. Of them, 39 were patients from couples who had been referred to the infertility center for examination and treatment, suffered oligoasthenoteratozoospermia (OAT), and the remaining 30 were included as controls (sperm donors) in a cross-sectional study design.

All patients were treated at the Department of Andrology from May 2017 to November 2017.

Patients with sperm concentration $< 15 \times 10^6$/mL, total motility < 40%, and > 4% morphologic anomalies, as confirmed by at least two tests, were considered to have OAT and were included in the study. Sperm donors with normozoospermia and proven fertility were included as fertile controls.

The study was performed according to the Declaration of Helsinki for Medical Research Involving Human Patients and approved by the local research ethics committee (Hospital de la Princesa, Madrid, approval n° PI-827). All participating subjects provided written informed consent.

Analysis of Semen Parameters

Semen samples were collected by masturbation after sexual abstinence for 2–5 days and kept at 37 °C for 30 min. After liquefaction, the volume of each semen sample was measured, followed by routine analysis. Sperm concentration, total motility, progressive motility (PR), and morphology were analyzed in accordance with the World Health Organization guidelines [29]. For each specimen, at least 200 spermatozoa were analyzed for each replicate. The remaining semen samples were centrifuged at 3000 g for 10 min, and the supernatants obtained were stored at − 20 °C until required for the afamin analysis.

Serum Sample Collection

Blood was obtained by conventional venipuncture, avoiding venous stasis. Whole-blood samples were collected in tubes with anticoagulated EDTA (ethylenediaminetetraacetic acid) and centrifuged at 3000 x g for 10 min. Thereafter, the plasma was aspirated with care to avoid the band of white globules situated between the plasma and the red globules. Then, red globule lysis solution was added, and the sample was centrifuged again at 3000 x g for 10 min. The supernatant was withdrawn by aspiration, and the process was repeated twice to avoid any traces of red globules. The leucocytes were kept at − 80 °C until analysis. The blood sample was drawn the same day that the semen sample was produced. Serum was stored at − 80 °C until analysis. Sera were then thawed and analyzed for afamin concentration.

Study Protocol

Measurements of Afamin

Afamin was quantified by double-antibody sandwich ELISA using an affinity-purified biotinylated polyclonal anti-afamin antibody for coating 96-well streptavidin-bound microtiter plates according to the manufacturer's procedure (CSB-EL001419HU, Cusabio).

Briefly, an antibody specific for AFM was precoated onto a microplate. Standards and samples were pipetted into the wells, and any AFM present was bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for AFM was added to the wells. After washing, avidin-conjugated horseradish peroxidase (HRP) was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells, and color developed in proportion to the amount of AFM bound in the initial step. The color development was stopped, and the intensity of the color was measured in a microplate reader at 450 nm. AFM concentrations from the sample are quantitated by interpolating absorbance readings from a standard curve generated with the calibrated afamin protein standard provided. The detection range was 3.9 ng/ml–250 ng/ml, and the minimum detectable dose of human AFM in the assay (sensitivity) was typically less than 0.97 ng/ml.

Secondary plasma in serial dilutions initially calibrated with a primary standard served as the assay standard. Afamin purified to homogeneity from human plasma was used as the primary standard; its exact protein concentration was determined by quantitative amino acid compositional analysis. Afamin serum and seminal plasma concentrations were quantified in duplicate, and the intra-assay coefficient of variance was 4.2%.

DNA Extraction

For DNA extraction, the FlexiGene DNA kit (Qiagen) was used in accordance with the manufacturer's directions, as follows: 10 ml Buffer FG1 (lysis buffer) was pipetted into a 50 ml centrifuge tube with 4 ml whole blood, mixed and centrifuged at 3600 rpm in a swing-out rotor in a Beckman GS-R5 centrifuge at 10 °C for 7 min. The supernatant was discarded, and then 2 ml of Buffer FG2/QIAGEN Protease mixture was added and vortexed immediately until the pellet was completely homogenized. This step efficiently removes contaminants such as proteins. These tubes were placed in a water bath and incubated at 65 °C for 10 min. The color of the sample changed from red to olive green, indicating protein digestion. Then, the DNA is precipitated and becomes visible as threads by pipetting 2 ml of isopropanol (100%). To purify the sample, it was washed with 70% ethanol and finally resuspended in 0.5 ml of Buffer FG3 (hydration buffer) by incubating for 1 h at 65 °C in a water bath. The concentration of the extracted DNA was determined using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Braunschweig, Germany) before dilution to a concentration of 50 ng/μl and storage at 4 °C. The purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A260/A280 ratio of 1.7–1.9.

Genotyping

Peripheral blood samples from patients and controls were analyzed for the presence of specific sequences of the afamin gene (AFM) by PCR amplification followed by direct sequencing. We studied the association between single-nucleotide polymorphisms (SNPs) in the 5`-untranslated region (5`-UTR) of the afamin gene and infertility. Polymorphisms in the promoter region of AFM were genotyped by sequencing. We analyzed a total of 1000 base pairs from the untranslated region of the afamin gene. Three pairs of primers were designed covering 1 kb of the 5′ sequence upstream of the afamin ORF obtained from the Ensemble web page (ENST00000226355). For PCR, we utilized the

following primers: 5′-CAA CCC TGC TGT GGA CCA C-3′ and 5′-GCA CAT ATG TTT TAT CAG CTT T-3′.

We then performed a more exhaustive study by searching the *Ensemble genomic database* (www.ensembl.org) to determine the allelic frequency of each variant that had been previously observed in genetic studies in the Iberian population for comparison with the results of our study.

Each reaction was prepared to a final volume of 25 μl containing 12.5 μl of GoTaq®G2 Hot Start Green master mix, 1 μl of each primer at 10 mM, 1 μl of DNA, and 9.5 μl of H_2O. The reaction was prepared in a thin-wall PCR microcentrifuge tube and gently centrifuged to collect all components to the bottom. Amplification was performed on a thermal cycler using the following program: denaturation at 95 °C for 5 min; 35 cycles at 95 °C for 1 min; 64 °C for 1 min; and 72 °C for 1.5 min; and 72 °C for 10 min. The PCR products were electrophoresed on an ethidium bromide-stained 2% agarose gel.

PCR amplicons were purified by using ExoSAP-IT (Affymetrix USB) according to the manufacturer's instructions. Specifically, 1.5 μl of ExoSAP-IT for PCR Product Cleanup (Affymetrix USB) was mixed with 4.5 μl of the post-PCR product and then incubated in the thermocycler for 15 min at 37 °C for enzyme activation and 15 min at 80 °C for enzyme inactivation.

Sequencing was performed with the same primer set using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Purified PCR product (6 μl) was mixed with 1 μl of the corresponding primer, 1 μl of Big Dye enzyme, and 2 μl of sequencing buffer. The program starts with 3 min at 98 °C; followed by 25 cycles of 10 s at 96 °C, 6 s at 50 °C, and 4 min at 60 °C; and a final hold at 12 °C. Two PCRs were sequenced for each sample, one in the forward sense and the other in the reverse sense, to obtain the full sequence. The results were analyzed using Chromas 2.4 software.

Statistical Analysis

Statistical analyses were performed with the SPSS software suite, version 17.0 (SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation, and the normal distribution of continuous variables was checked using Student's *t* test. One-way ANOVA was used for comparisons among groups. $p < 0.05$ was considered to indicate a statistically significant difference.

G*Power 3.1.2 (Kiel, Germany) was used to calculate the sample size needed for this study. Using a normal population median of serum afamin of 64 mg/L [7] and setting an α error probability of 0.05 and power (1-β error probability) of 0.80, we performed a two-tailed sample size calculation to detect a difference of 12% between OAT and normal subjects. A minimum of 30 subjects was needed to achieve adequate power to detect this difference.

Results

Patient Clinical Data

The clinical characteristics of the male study population are presented in Table 1 and include age, body mass index (BMI), semen volume (ml), total sperm count (10^6), sperm concentration (10^6), motility (%), and morphology (%). There were significant differences between male patients and controls in age ($p < 0.01$), total sperm count ($p < 0.001$), sperm concentration ($p < 0.001$), motility ($p < 0.01$), and morphology ($p < 0.01$).

Afamin Concentrations

Subjects with low sperm motility and/or low sperm concentration had higher median seminal afamin concentrations (18.9 ± 2.9 ng/mg of protein) and serum afamin concentrations (24.1 ± 4.0 ng/mg of protein) than those without sperm alterations (10.6 ± 1.4 ng/mg of protein) ($p < 0.02$; 15.6 ± 1.4 ng/mg of protein) ($p < 0.002$) (Table 1).

Using Pearson's parametric correlation test, we found a statistically significant negative correlation between semen afamin levels and the number of spermatozoa (Pearson's *r*: − 0.394, *p*: 0.001) and sperm motility (Pearson's *r*: − 0.310, *p*: 0.009).

Polymorphisms of Afamin Genes

Polymorphisms in the promoter region of AFM were genotyped by sequencing to identify the association between single-nucleotide polymorphisms (SNPs) in the 5`-untranslated region (5`-UTR) of the afamin gene and oligoasthenoteratozoospermia. One thousand base pairs from the untranslated region of the afamin gene were analyzed, and a total of five different polymorphisms were observed. One of them was a deletion, and the remaining four were considered single-nucleotide polymorphisms (SNPs). Table 2 shows a new transversion (A/T) (position 4:73481093) identified in an oligoasthenoteratozoospermia patient. In this new variant (variant A), the nucleotide T (thymine) was modified to become nucleotide A (adenine). The genotype prevalence at this site among patients in our study was 98.5% homozygous TT and 1.5% heterozygous TA.

As shown in Table 2, the determination of the allelic frequency observed in variant D (rs371710141) is also remarkable, since the frequency of this variant in the Iberian population had not been specified in previous studies. Our data show a prevalence of 98.6% for TT and 4.4% for TA.

Table 1 Characteristics of male patients ($n = 39$) and fertile donors ($n = 30$) in terms of age, body mass index, semen volume (ml), total sperm count (10^6), sperm concentration (10^6), motility (%), morphology (%), and level of afamin in serum and seminal plasma

Mean ± SD	Male patients ($n = 39$)	Sperm donors ($n = 30$)	p value
Age (years)	39.5 ± 10	21.0 ± 2	**0.01**
Body mass index	23.9 ± 4.3	21.5 ± 3.3	0.73
Semen volume (ml)	3.2 ± 1.8	2.85 ± 1.5	0.26
Total sperm count (× 10^6)	34 ± 3.5	138 ± 17	**0.001**
Sperm concentration/ml(× 10^6)	11.2 ± 4.1	87 ± 15	**0.001**
Motility (%)	16.4 ± 6	62.6 ± 10	**0.01**
Normal morphology (%)	3.2 ± 1.6	12.5 ± 5	**0.01**
Afamin serum *(ng/mg of proteins)*	24.1 ± 4.03	10.6 ± 1.39	**0.002**
Afamin semen *(ng/mg of proteins)*	18.8 ± 2.9	10.58 ± 1.37	**0.002**

There were significant differences between the male study population and controls in age (< 0.01), total sperm count ($p < 0.001$), sperm concentration ($p < 0.001$), motility ($p < 0.01$), morphology ($p < 0.01$), and total number of motile sperm recovered

Subjects with low sperm motility and/or sperm concentration had higher median seminal afamin (18.9 ± 2.9 ng/mg of protein) and serum afamin concentrations (24.1 ± 4.0 ng/mg of protein) than those without sperm alterations (10.6 ± 1.4 ng/mg of protein) ($p < 0.02$; 15.6 ± 1.4 ng/mg of protein) ($p < 0.002$)

Values are given as the mean ± standard deviation (SD). Statistically significant p values are shown as bold numbers

The remaining analyzed variants are in consonance with those described in the Iberian population.

Figure 1 shows the sequence 1000 base pairs upstream (5′) of the UTR region of the afamin gene with the detected polymorphisms indicated in different colors.

Single-Nucleotide Polymorphisms (SNPs) and Afamin Levels

The frequencies of SNPs between the patient and control (donor) groups and their relationships with the level of afamin were compared. The new transversion (A/T) (position 4:73481093) was identified in an OAT patient and was associated with high levels of afamin in plasma and seminal fluids. The sequence profile of this OAT patient carrying the new variant is shown in Fig. 2.

As shown in Table 3, the 5′ upstream single-nucleotide polymorphisms rs35680917, rs115041046, rs371710141, and rs72856618 of AFM were polymorphic in both the patient and control groups, and the TA genotype (rs371710141) was predominant in the male patients.

Special attention should be given to rs115041046 (variant C), as we observed that patients present a heterozygous variant for AG (14.3% of patients) that we did not find in the control group (Table 3).

Discussion

In the present study, we assessed the level of afamin in the seminal plasma and serum of 39 male infertility patients and 30 healthy controls and investigated the genetic factors that might contribute to the determination of afamin activity. Our results overall suggest that the afamin levels in both the semen and serum were higher in patients with oligoasthenoteratozoospermia than in normozoospermic males, which implies that afamin has a role in male fertility. Moreover, we found a correlation between the levels of afamin in plasma and in semen, which has not been studied previously.

Afamin was biochemically characterized as a vitamin E–binding protein [3, 4]. The role of tocopherol (vitamin E) in the prevention of the damage caused by free radicals and lipid peroxidation is well established [30], and it is conceivable that the level of afamin is a marker of oxidative stress requiring the presence of vitamin E. Vitamin E, as a lipid-soluble antioxidant, can restore oxidizing radicals directly, preventing the chain propagation of lipid peroxidation, thus playing a vital role in protecting cell membranes [31]. Additionally, there is some evidence to support a fundamental role of oxidative stress in the etiology of male infertility through negative effects on sperm quality and function [32]. This presumption is supported by the observation that vitamin E levels can also be altered in certain pathologies related to the reproductive system, as indicated by various previous studies. For instance, Bhardwaj et al. [33] found reduced vitamin E levels and reduced glutathione in the semen of oligozoospermic and azoospermic patients. Al Alzemi and colleagues, in 2009 [34], observed that the concentrations of α-tocopherol and retinol (or vitamin A) in men with normal seminal parameters were significantly higher than those in men with oligozoospermia and asthenozoospermia. Similarly, Zerbinati et al. [35] demonstrated the relationship between alpha- and gamma-tocopherol levels in human semen and male infertility. Benedetti et al. [36] found that the total antioxidant capacity and vitamin E concentrations in seminal plasma and blood samples were

Table 2 Variant descriptions indicating for each variant the rs number, identification (ID), alleles, Iberian population in Spain (IBS) allele frequencies, and observed allele frequencies

Variant	Alleles	Location	Allelic Frequency IBS	Allelic Frequency Observed
New Variant (A)	A/T	4:73481093	–	T|T: 0.985 T|A: 0.015
(B) rs35680917	TG/–	4:73481197–73481198	TG|TG: 0.393 -|TG: 0.486 -|-: 0.121	TG|TG: 0.544-|TG: 0.397 -|-: 0.059
(C) rs115041046	A/G	4:73481316	G|G: 1.000	G|G: 0.971 G|A: 0.029
(D) rs371710141	T/A	4:73481344	–	T|T: 0.956 T|A: 0.044
(E) rs72856618	G/T	4:73481499	T|T: 0.888 G|T: 0.112	T|T: 0.897 G|T: 0.103

New variant (variant A) where the nucleotide T (thymine) was modified to become nucleotide A (adenine). The prevalence of this variant in our study was 98.5% homozygous TT and 1.5% heterozygous TA

Allelic frequency in variant D (rs371710141): 98.6% homozygous TT and 4.4% heterozygous TA

significantly lower in infertile men than in fertile subjects. These results indicated the possibility of using not only seminal antioxidants but also blood antioxidants as biochemical markers to support sperm quality evaluation.

Notably, our results show that the level of afamin is higher in infertile patients. The finding of greater amounts of afamin in OAT patients appears contradictory to certain publications indicating that afamin is required for normal growth and

Fig. 1 Sequence of 1000 base pairs upstream (5′) of the UTR region of the afamin gene with the detected polymorphisms indicated in different colors. After analyzing a total of 1000 base pairs from the untranslated region of the afamin gene, a total of five different polymorphisms were detected by sequencing. One of them was a deletion, and the remaining four were considered single-nucleotide polymorphisms (SNPs)

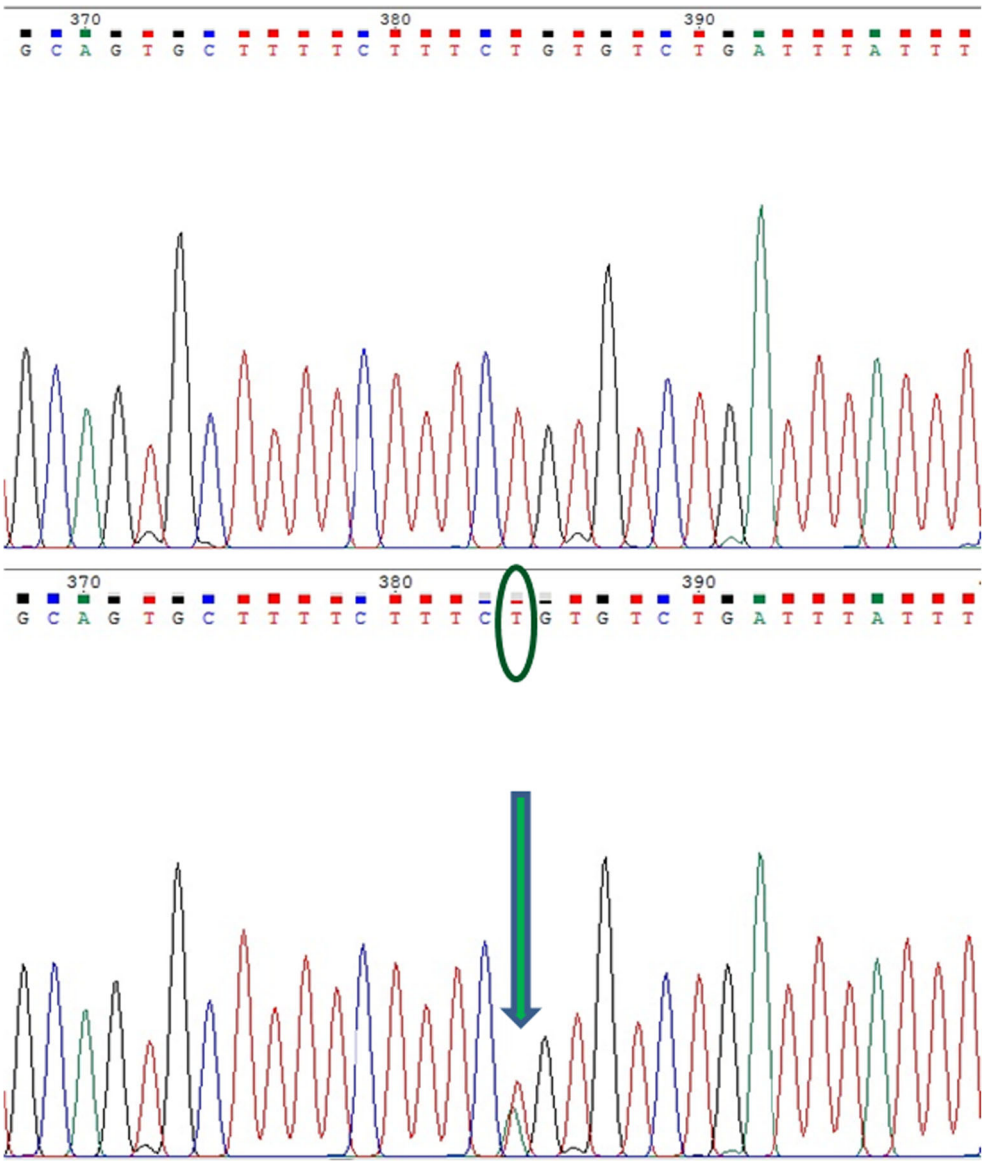

Fig. 2 Sequence chromatogram from an oligoasthenoteratozoospermic (OAT) patient carrying the new variant. A fragment of the AFM gene indicating the position of the nucleotide substitution (arrow) corresponding to the c.384 T mutation is shown. In this new variant (variant A) (not identified to date), the nucleotide T (thymine) was modified to become nucleotide A (adenine). The prevalence of this variant in OAT cases was 98.5% homozygous TT and 1.5% heterozygous TA. The new transversion (A/T) (position 4: 73481093) was identified in an OAT patient and was associated with high levels of afamin in plasma and seminal fluid

health of male reproductive organs [27]. We hypothesize that the higher afamin content may reflect a compensatory mechanism caused by a possible decrease in vitamin E levels and an increase in oxidative stress. This is, however, mainly speculative, and further studies are necessary to clarify this issue since we did not measure serum vitamin E levels in the current study. Another explanation could be that, in general, afamin has heterogeneous effects depending on the site of action [27].

Nevertheless, our results are consistent with those published by Beata et al. [9], who found higher levels of afamin in stage I and II endometriosis. The authors assumed that the greater afamin levels might be due to a more acute stage of disease and signify an active recruitment of antioxidants, including vitamin E, to this local inflammatory environment. Other studies also show a relationship between pathologies such as diabetes [25] and preeclampsia [10] and increased plasma levels of afamin. In line with these findings, elevated afamin concentrations were demonstrated in patients with polycystic ovary syndrome, which is associated with insulin resistance [37].

On the other hand, since afamin has been associated with metabolic syndrome (24), we considered the body mass index in the two study groups. No statistically significant difference in BMI was found between the two groups.

Although several studies have identified afamin in different diseases, as mentioned above, studies of afamin in male infertility are scarce. To the best of our knowledge, our work is the first report investigating the afamin protein and its relationship with male infertility. This is important, since despite decades of research on this protein,

Table 3 Allele and genotype distributions of five single-nucleotide polymorphisms of afamin in infertile patients and controls

Locus	Genotype/ allele	Controls ($n = 30$)	Patients ($n = 39$)
New variant (A)			
	TT	100%	85.7%
	TA	0	14.3%
(B) rs35680917			
	TGTG	52.6%	42.9%
	TG	36.8%	57.1%
	–	10.6%	0
(C) rs115041046			
	GG	100%	85.7%
	AG	0	14.3%
(D) rs371710141			
	TT	94.7%	85.7%
	TA	5.3%	14.3%
(E) rs72856618			
	TT	89.4%	85.7%
	GT	10.6%	14.3%

5′ upstream single-nucleotide polymorphisms rs35680917, rs72856618, rs115041046, rs371710141, and rs72856618 of AFM were polymorphic in both the patient and control groups

The AG genotype (rs115041046) (variant C) was more frequent among patients than among the control group, and the TA genotype (rs371710441) (variant D) was predominant in the group of male patients

our knowledge of the pathophysiology of afamin is limited.

Furthermore, we studied the association between single-nucleotide polymorphisms (SNPs) in the promoter region of the afamin gene and infertility. Our study identified a new transversion (A/T) (position 4:73481093) in an oligoasthenoteratozoospermia patient associated with high levels of afamin in plasma and seminal fluids. This finding indicates that the mentioned polymorphism may be linked to susceptibility to oligoasthenozoospermia. Thus, we speculate that higher afamin activity in seminal plasma caused by the new transversion (A/T) (position 4:73481093) may result in male infertility.

Additionally, comparing variant genotypes between patients and donors, we found that in rs115041046 (variant C), several OAT patients presented a heterozygous variant for AG (14.3%), which we did not find in the control group. Moreover, the TA genotype of rs371710141 was predominant in the group of male patients.

These findings are supported by studies showing that genetic polymorphisms may increase susceptibility to some forms of male infertility [38].

However, our understanding of the regulation of the afamin gene remains limited. Only a microarray study by Liu et al. [39] showed increased expression of the transcription factor islet-1 (which is associated with increased β-cell function) to increase afamin expression 35-fold.

In this study, the number of cases and controls was relatively small, so the results should be considered preliminary; further research in larger cohorts could explore afamin cutoff values indicating a pathological diagnosis in male infertility.

Another limitation in our study is the significant age difference between patients and controls. However, a previous study reported no age dependency of afamin concentrations in a large group of healthy blood donors [7].

Conclusions

The main findings of this study were as follows: (1) increased afamin serum and semen concentrations were significantly associated with oligoasthenoteratozoospermia and (2) afamin genetic variations might be associated with male infertility.

These findings could significantly enhance our understanding of the molecular genetic causes of infertility. The causality of the association of afamin with male infertility, as well as possible underlying mechanisms, remains to be elucidated.

Authors' Contributions RNC contributed to the conception and design of the study, data analysis, interpretation of the data, and drafting of the manuscript. SC was responsible for the collection of sperm samples and aided in drafting the manuscript. PCP was responsible for data analysis and interpretation. LMGG and RK were responsible for the afamin measurements, DNA extraction, and genotyping. RK was responsible for revision and final approval of the manuscript.

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Compliance with Ethical Standards

The study was allowed by ethical committee: Hospital de la Princesa, Madrid, approval n° PI-827.

Conflict of Interest The authors declare they have no competing interests.

References

1. Lichenstein HS, Lyons DE, Wurfel MM. Afamin is a new member of the albumin, α-fetoprotein, and vitamin D-binding protein gene family. J Biol Chem. 1994;269:18149–54.
2. Bélange J, Roy D, Allard D. New albumin gene 3′ adjacent to the α1-fetoprotein locus. J Biol Chem. 1994;269:5481–4.
3. Jerkovic L, Voegele AF, Chwatal S. Afamin is a novel human vitamin E-binding glycoprotein characterization and in vitro expression. J Proteome Res. 2005;4:889–99.
4. Voegele AF, Jerkovic L, Wellenzohn B. Characterization of the vitamin E-binding properties of human plasma afamin. Biochemistry. 2002;41:14532–8.
5. Angelucci S, Ciavardelli D, Di Giuseppe F. Proteome analysis of human follicular fluid. Biochim Biophys Acta. 2006;1764:1775–85.

6. Heiser M, Hutter-Paier B, Jerkovic L, Pfragner R, Windisch M, Becker-Andre M, et al. Vitamin E binding protein afamin protects neuronal cells in vitro. J Neural Transm Suppl. 2002;62:337–45.
7. Dieplinger H, Dieplinger B. Afamin–a pleiotropic glycoprotein involved in various disease states. Clin Chim Acta. 2015;15(446): 105–10.
8. Lambrinoudaki IV, Augouela A, Christodoulakos GE, Economou EV, Kaparos G, Kontoravdis A. Measurable serum markers of oxidative stress response in women with endometriosis. Fertil Steril. 2009;91:46–50.
9. Seeber BE, Czech T, Buchner H, Barnhart KT, Seger C, Daxenbichler G, et al. The vitamin E–binding protein afamin is altered significantly in the peritoneal fluid of women with endometriosis. Fertil Steril. 2010;94:2923–6.
10. Köninger A, Enekwe A, Mach P, Andrikos D, Schmidt B, Frank M, et al. Afamin: an early predictor of preeclampsia. Arch Gynecol Obstet. 2018;298:1009–16.
11. Köninger A, Mathan A, Mach P, Frank M, Schmidt B, Schleussner E, et al. Is Afamin a novel biomarker for gestational diabetes mellitus? A pilot study. Reprod Biol Endocrinol. 2018;27:30–8.
12. Ringman JM, Schulman H, Becker C, Jones T, Bai Y, Immermann F, et al. Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer disease mutations. Arch Neurol. 2012;69:96–104.
13. Rosenling T, Stoop MP, Attali A, Aken H, Suidgeest E, Christin C, et al. Profiling and identification of cerebrospinal fluid proteins in a rat EAE model of multiple sclerosis. J Proteome Res. 2012;11: 2048–60.
14. Song F, Poljak A, Kochan NA, Raftery M, Brodaty H, Smythe GA, et al. Plasma protein profiling of mild cognitive impairment and Alzheimer's disease using iTRAQ quantitative proteomics. Proteome Sci. 2014;12:5.
15. Jackson D, Craven RA, Hutson RC. Proteomic profiling identifies afamin as a potential biomarker for ovarian cancer. Clin Cancer Res. 2007;13:7370–9.
16. Dieplinger H, Ankerst DP, Burges A. Afamin and apolipoprotein A-IV: novel protein markers for ovarian cancer. Cancer Epidemiol Biomark Prev. 2009;18:1127–33.
17. Melmer A, Fineder L, Lamina C. Plasma concentrations of the vitamin E-binding protein afamin are associated with overall and progression-free survival and platinum sensitivity in serous ovarian cancer—a study by the OVCAD consortium. Gynecol Oncol. 2013;128:38–43.
18. Chen YT, Chen HW, Domanski D, Smith DS, Liang KH, Wu CC, et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J Proteome. 2012;75: 3529–45.
19. Choi JW, Liu H, Shin DH, Yu GI, Hwang JS, Kim ES, et al. Proteomic and cytokine plasma biomarkers for predicting progression from colorectal adenoma to carcinoma in human patients. Proteomics. 2013;13:2361–74.
20. Humphries JM, Penno MA, Weiland F, et al. Identification and validation of novel candidate protein biomarkers for the detection of human gastric cancer. Biochim Biophys Acta. 1844;2014:1051–8.
21. Jeong DH, Kim HK, Prince AE, et al. Plasma proteomic analysis of patients with squamous cell carcinoma of the uterine cervix. J Gynecol Oncol. 2008;19:173–80.
22. Wang WK, Tsai CH, Liu YW, Lai CC, Huang CC, Sheen-Chen SM. Afamin expression in breast cancer. Asian J Surg. 2020;43: 750–4.
23. Song HJ, Xue YL, Qiu ZL, Luo QY. Comparative serum proteomic analysis identified afamin as a downregulated protein in papillary thyroid carcinoma patients with non-131I-avid lung metastases. Nucl Med Commun. 2013;34:1196–203.
24. Kronenberg F, Kollerits B, Kiechl S, Lamina C, Kedenko L, Meisinger C, et al. Plasma concentrations of afamin are associated with the prevalence and development of metabolic syndrome. Circ Cardiovasc Genet. 2014;7:822–9.
25. Kollerits B, Lamina C, Huth C, Marques-Vidal P, Kiechl S, Seppälä I, et al. Plasma concentrations of afamin are associated with prevalent and incident type 2 diabetes: a pooled analysis in more than 20, 000 individuals. Diabetes Care. 2017;40:1386–93.
26. Kaburagi Y, Takahashi E, Kajio H, Yamashita S, Yamamoto-Honda R, Shiga T, et al. Urinary afamin levels are associated with the progression of diabetic nephropathy. Diabetes Res Clin Pract. 2019;147:37–46.
27. https://patents.google.com/patent/EP2328605A1/en.
28. Liu J, Huang X, Cao X, Feng X, Wang X. Serum biomarker analysis in patients with premature ovarian insufficiency. Cytokine. 2020;126:154876.
29. World Health Organization. Laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010.
30. Asbaghi O, Sadeghian M, Nazarian B, Sarreshtedari M, Mozaffari-Khosravi H, Maleki V, et al. The effect of vitamin E supplementation on selected inflammatory biomarkers in adults: a systematic review and meta-analysis of randomized clinical trials. Sci Rep. 2020;10(1):17234.
31. Zhang JG, Nicholls-Grzemski FA, Tirmenstein MA, Fariss MW. Vitamin E succinate protects hepatocytes against the toxic effect of reactive oxygen species generated at mitochondrial complexes i and iii by alkylating agents. Chem Biol Interact. 2001;138(3): 267–84.
32. Agarwal A, Rana M, Qiu E, AlBunni H, Bui AD, Henkel R. Role of oxidative stress, infection, and inflammation in male infertility. Andrologia. 2018;50:131–6.
33. Bhardwaj A, Verma A, Majumdar S, Khanduja KL. Status of vitamin E and reduced glutathione in semen of oligozoospermic and azoospermic patients. Asian J Androl. 2000;2(3):225–8.
34. Al-Azemi MK, Omu AE, Fatinikun T, Mannazhath N, Abraham S. Factors contributing to gender differences in serum retinol and alpha-tocopherol in infertile couples. Reprod BioMed Online. 2009;19(4):583–90.
35. Zerbinati C, Caponecchia L, Fiori C, Sebastianelli A, Salacone P, Ciacciarelli M. Iuliano L alpha- and gamma-tocopherol levels in human semen and their potential functional implications. Andrologia. 2020;52:56–62.
36. Benedetti S, Tagliamonte MC, Catalani S, Primiterra M, Canestrari F, De Stefani S, et al. Differences in blood and semen oxidative status in fertile and infertile men, and their relationship with sperm quality. Reprod BioMed Online. 2012;25(3):300–6.
37. Koninger A, Edimiris P, Koch L, Enekwe A, Lamina C, Kasimir-Bauer S, et al. Serum concentrations of afamin are elevated in patients with polycystic ovary syndrome. Endocr Connect. 2014;3(3):120–6.
38. O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. Fertil Steril. 2010;93:1–12.
39. Liu J, Walp ER, May CL. Elevation of transcription factor Islet-1 levels in vivo increases β-cell function but not β-cell mass. Islets. 2012;4(3):199–206.