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Population-genetic and molecular-biological studies in patients with hereditary transthyretin amyloidosis in Bulgaria

ABSTRACT OF PHD THESIS

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The official defense of the dissertation work will take place on 24.04.2023. at the Department of Medical Chemistry and Biochemistry, Medical University - Sofia, 2 Zdrave St., Sofia, before a scientific jury composed of:

1. Prof. Alexey Slavkov Savov, PhD (review)
2. Assoc. Prof. Lubomir Lyubomirov Traikov, PhD (opinion)
3. Assoc. Prof. Maria Borisova Ivanova, PhD (opinion)
4. Prof. Svetla Dimitrova Petrova, PhD (review)
5. Prof. Radoslava Vasileva Vazharova, PhD (opinion)

The materials regarding the dissertation defense are available at the Department of Medical Chemistry and Biochemistry, Medical University - Sofia.

CONTENT

ABBREVIATIONS

ATTRv – hereditary transthyretin amyloidosis

ATTRwt – wild type transthyretin amyloidosis

TTR-FAP – transthyretin familial amyloid polyneuropathy

TTR-FAK – transthyretin familial amyloid cardiomyopathy

DBS – dried blood spot sample

PCR – polymerase chain reaction

SNP – single nucleotide polymorphism

EDTA – ethylenediaminetetraacetic acid

VUS - variant of unknown clinical significance

cDNA – copy DNA

ELB - Excoffier-Laval-Balding algorithm

MCMC - Markov chain Monte Carlo algorithm

SUMMARY

ATTRv is a late-onset multisystem genetic condition affecting the peripheral nervous system, the autonomic nervous system, the heart, etc. It is characterized by certain genetic and phenotypic heterogeneity. Bulgaria is among the countries with a relatively high frequency of disease-causing amyloidogenic variants in the *TTR* gene. In Bulgaria, the epidemiologic prevalence of the common *TTR* pathogenic variants, with Glu89Gln being the most frequent, is to great extend endemic. The research carried out within this dissertation covers various aspects regarding the prevalence, clinical manifestation and molecular mechanisms of pathogenesis in patients with hereditary transthyretin amyloidosis in Bulgaria.

Screening for pathogenic variants in the *TTR* gene was performed in suspected new patients and a new variant for our population, known as Glu54Leu was added to the list of *TTR* amyloidogenic variants in Bulgaria. Relatives of the newly diagnosed patients were also genetically tested, where the high proportion of positive asymptomatic carriers among the study group proved the need for genetic screening among the affected families. The negative results from the screening of Roma newborns from the region of the cities of Polski Trumbesh and Ruse rejected the hypothetical endemicity of the Gly47Glu variant, leaving the variants for which there are proven endemic regions in Bulgaria to be Glu89Gln, Val30Met and Ser77Phe. The results from the genetic screening for carriers of pathogenic variants confirmed the presence of a hot region in exons 2 and 3 of the *TTR* gene for the accumulation of amyloidogenic variants in the Bulgarian population.

Genotyping of frequent polymorphisms with a potential disease-modifying effect was performed in patients with the *TTR* variant Glu89Gln, the results of which showed the presence of a statistical correlation between the age at onset and the rs1791228 polymorphism. Analysis of the effect of rs1791228 on initial system involvement in the studied cohort also showed an increase in the proportion of patients carrying the T allele with initial cardiac and mixed involvement. The observed results are concordant with the data reported in the literature, but the proposed molecular mechanisms are not yet sufficiently studied to make a reliable conclusion about the effect of this polymorphism on the development of hereditary transthyretin amyloidosis.

The results from the analysis of the transthyretin gene expression in urine and blood plasma samples of Glu89Gln patients revealed a mixed mono- and biallelic transcriptional profile with different ratios between mutant and wild-type transthyretin, which could hypothetically have an effect on disease progression. Based on the analysis of two monozygotic twins, a hypothetical mechanism for age-dependent allele-specific gene expression was proposed. Although more research is needed to clarify the relationship between the observed specific expression profile and the development of hereditary transthyretin amyloidosis, foundations have been laid for studies on molecular prognostic markers of disease progression.

Haplotype analysis of microsatellite markers linked to the *TTR* gene was performed and the results statistically confirmed the hypothesis for an existing founder effect for the *TTR* variants Glu89Gln, Val30Met, Ser77Phe and Gly47Glu in Bulgaria. The theoretical age of the most recent common ancestor for the four variant groups was calculated, finding that Val30Met arose most distantly into our population. The results of the population-genetic studies showed that the Bulgarian hereditary transthyretin amyloidosis patients possess a unique genetic profile, which is a potential background for future research on the still understudied heterogeneity in the disease phenotype and clinical manifestation.

INTRODUCTION

Amyloidosis is a group of diseases, most often with an onset in adulthood, which are caused by an abnormal accumulation of protein aggregates in various tissues and organs. The pathological effect is specific to the disease-causing protein. There are over 40 different types of amyloidosis, one of which is transthyretin amyloidosis. It is caused by the accumulation of amyloid fibrils composed of the protein transthyretin and occurs in two forms: wild-type transthyretin amyloidosis and hereditary transthyretin amyloidosis, which is caused by pathogenic variants in the *TTR* gene.

Bulgaria is among the countries with a relatively high frequency of hereditary transthyretin amyloidosis. Clinical-genetic studies in the field began more than 10 years ago in our country, and a significant patient data base has already been accumulated with more than 100 families carrying pathogenic variants in the *TTR* gene.

The current dissertation focuses on research of the Bulgarian cohort of patients with hereditary transthyretin amyloidosis in terms of the genetic and phenotypic heterogeneity, the molecular mechanisms behind the differences in the clinical manifestation of the disease, as well as research of the genealogy in the endemic regions of the country.

1. AIMS AND OBJECTIVES

1.1. Aims

- Study of genetic heterogeneity, the effect of genetic modifiers and gene expression on phenotypic heterogeneity in patients with hereditary transthyretin amyloidosis in Bulgaria.
- Assessment of the founder effect regarding the most common pathogenic variants in the *TTR* gene.

1.2. Objectives

- Search for pathogenic variants in the *TTR* gene for complete genetic profiling of hereditary transthyretin amyloidosis in Bulgaria.
- Neonatal screening for the pathogenic *TTR* variant Gly47Glu among newborns of Roma origin.
- Study of the association between genetic polymorphisms with probable regulatory function and differences in age at onset and system involvement in carriers of the pathogenic *TTR* variant Glu89Gln.
- Transthyretin expression analysis on carriers of the pathogenic *TTR* variant Glu89Gln.
- Examination of genetic markers near the *TTR* gene to investigate a common haplotype in the carriers of the same pathogenic variant.
- Theoretical determination of the age of the most recent common ancestor in carriers of the same pathogenic variant in the *TTR* gene.

2. MATERIALS AND METHODS

2.1. Clinical and biological material

In the current dissertation, a total of 586 samples were examined and their distribution according to the study stage is described in Table 1.

Table 1. Summary of tested samples by stages

	Study stage		Number of tested samples
1.	Screening for pathogenic variants in the *TTR* gene	Screening in suspected new patients - venous blood	59
		Screening in relatives of the proven ATTRv patients - venous blood	23
		Screening in suspected patients, who are negative for pathogenic variants in exons 2 and 3 of the *TTR* gene - genomic DNA from venous blood	56
		Neonatal screening to determine the carrier frequency of the *TTR* variant Gly47Glu among Roma newborns- DBS	100
2.	Analysis of regulatory genetic polymorphisms with potential effect on the clinical manifestation of the disease - genomic DNA from venous blood of Glu89Gln ATTRv patients.		124
3.	Expression analysis of the *TTR* gene - total RNA isolated from urine and/or blood plasma of Glu89Gln ATTRv patients.		19
4.	Haplotype analysis to determine founder effect (ATTRv patients, healthy relatives and controls) - genomic DNA from venous blood.		205
		Total number of samples	**586**

2.2. Methods

- Extraction of high molecular weight DNA from peripheral blood by desalting method
- Extraction of high molecular weight DNA from DBS
- Extraction of RNA from blood plasma and urine
- Sanger sequencing
- Statistical analysis for correlation - Pearson's Chi-square test
- RNA sequencing of the TTR gene
- Quantification of allele-specific transthyretin expression – allele-specific cDNA PCR and fragment analysis
- Microsatellite fragment analysis
- Haplotype reconstruction - ELB algorithm
- Determination of "mutation age" - MCMC algorithm

3. RESULTS AND DISCUSSION

3.1. Screening for pathogenic *TTR* variants

3.1.1. Screening in patients

The results of the screening in index patients with a suspected diagnosis of ATTRv are shown in Table 2. In addition to the previously known pathogenic variants Glu89Gln, Val30Met and Ser77Phe, a new for Bulgaria amyloidogenic variant c.220_221delGAinsCT, p.Glu74Leu (Glu54Leu) was detected. Another genetic variant c.14G>A, p.Arg5His, new for the Bulgarian population, was also found, which was classified as a variant of unknown clinical significance.

Table 2. Results of the genetic testing in index patients referred for *TTR* gene sequencing. Variants are represented according to the coding sequence of transcript NM_000371.4(TTR).

Index patients		
Positive	c.325G>C, p.Glu109Gln (Glu89Gln)	5
	c.148G>A, p.Val50Met, (Val30Met)	1
	c.290C>T, p.Ser97Phe (Ser77Phe)	1
	c.220_221delGAinsCT, p.Glu74Leu (Glu54Leu)	1
	Total positive	**8**
Negative with VUS	c.14G>A, p.Arg5His	1
Negative		106
	Total number of tested patients	**115**

In the families of some of the positive ATTRv patients, it was possible to screen for asymptomatic carriers of the detected familial defect (Table 3). The results showed the presence of 12 positive relatives in these families.

Table 3. Results of the genetic testing in relatives of ATTRv patients.

Relatives of ATTRv patients		
Positive	c.325G>C, p.Glu109Gln (Glu89Gln)	6
	c.148G>A, p.Val50Met, (Val30Met)	3
	c.220_221delGAinsCT, p.Glu74Leu (Glu54Leu)	3
	Total positive	**12**
Negative		11
	Total number of tested relatives	**23**

The available clinical data for the newly diagnosed *TTR* positive patients largely match the genotype-phenotype correlation known to date (Table 4). Patients with Glu89Gln show a mixed phenotype, simultaneously affecting the peripheral nervous system, cardiac functions, and gastrointestinal tract. The patient carrying the Ser77Phe variant also presented with peripheral polyneuropathy and cardiac amyloidosis, but has no gastrointestinal symptoms to date. The clinical manifestation in the patient with Val30Met is mainly neurological and with later age at onset of the disease.

Table 4. Clinical characteristics of ATTRv patients. The number of clinically described positive patients is shown in parentheses.

System affection	Glu89Gln (6)	Val30Met (1)	Ser77Phe (1)	Glu54Leu (2)
Neurological	Carpal tunnel syndrome Polyneuropathic sensory-motor syndrome Peripheral vestibular syndrome	Polyneuropathic sensory-motor syndrome Peripheral vestibular syndrome	Polyneuropathic sensory-motor syndrome Spinal canal stenosis	Carpal tunnel syndrome Polyneuropathic sensory-motor syndrome Lumbar vertebral syndrome
Cardiological	Amyloid cardiomyopathy Left ventricular hypertrophy		Amyloid cardiomyopathy	Amyloid cardiomyopathy Left ventricular hypertrophy
Gastrointestinal	Constipation Diarrhea Weight loss	Alternation of constipation and diarrhea		
Mean age at onset	49.8	66	56	47.5

The variant p.Glu74Leu (Glu54Leu) is new for Bulgaria and was found in only one family from Targovishte. The index patient was referred for genetic testing due to overt left ventricular hypertrophy with suspected cardiac amyloidosis. Bilateral carpal tunnel syndrome and polyneuropathic syndrome have been reported. A complex genetic variant representing a substitution of two adjacent nucleotides in exon 3 of the *TTR* gene was found in him (Figure 1). After performing a segregation analysis in the family, it was found that the two nucleotide substitutions are located on one allele (c.220_221delGAinsCT) and lead to the change of the negatively charged amino acid glutamate with the neutral amino acid leucine at position 74 of the amino acid sequence of transthyretin. The Glu54Leu variant has been reported in ATTRv patients from Sweden, Belgium and the UK[1], but has only been further described in one family from Sweden[2]. It is associated with severe cardiac and sometimes milder neurological involvement, and early age at onset. The altered genetic locus affects the D-strand of the β-sheet structure of the protein[3] and disrupts hydrophobic and other weak interactions, making it highly amyloidogenic[2] (Figure 2).

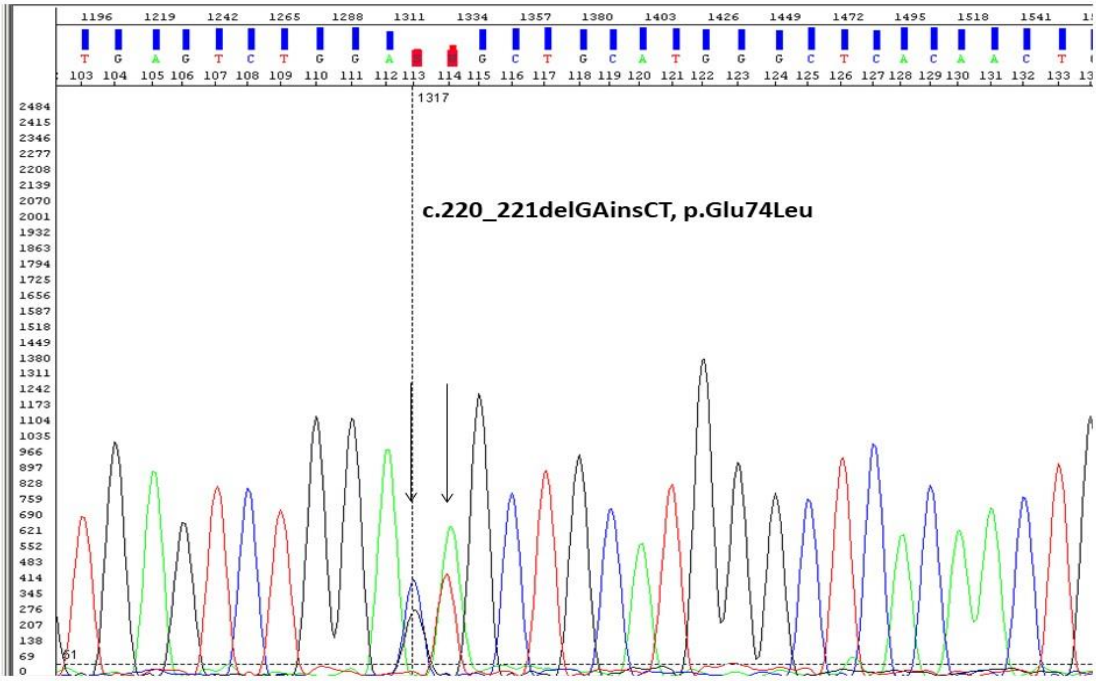

Figure 1. Sequencing electrophoregram of exon 3 of the *TTR* gene in the patient carrying the p.Glu74Leu (Glu54Leu) variant. The two adjacent nucleotide substitutions are indicated by arrows.

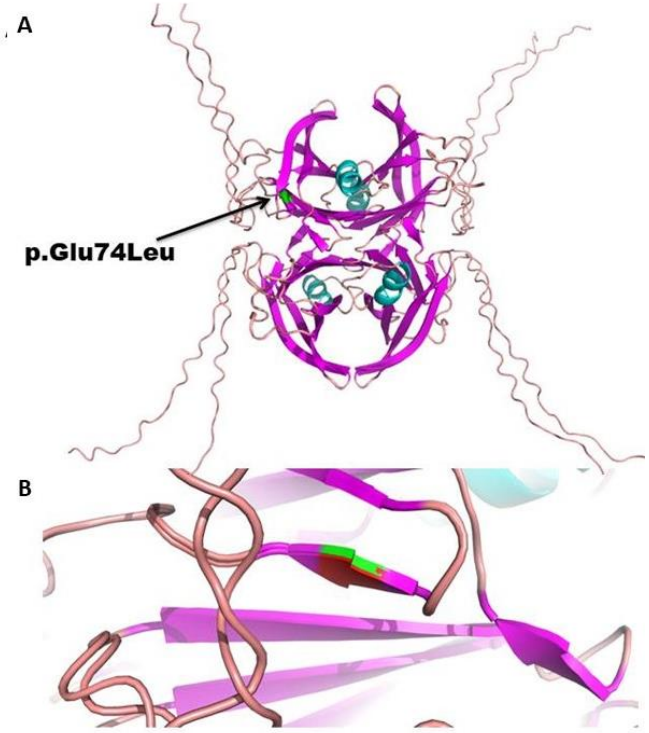

Figure 2. Protein model[4] illustrating the localization of the p.Glu74Leu (Glu54Leu) variant in the tetrameric transthyretin (А) and a magnification of the β-sheet of the monomeric protein (Б).

Data is available for at least 5 amyloidogenic pathogenic variants in codon 74 of the coding sequence of the *TTR* gene[5]. Glu54Leu occurs in two genetic variants that, due to the degeneracy of the genetic code, result in the same amino acid substitution (c.220_221delGAinsCT and c.220_221delGAinsTT). The c.220_221delGAinsTT variant has been reported once in a patient from Belgium and once in a patient from Scotland[6]. Of particular importance here is the correct nomenclatural reporting of the discovered new genetic variants in scientific publications and databases, so that the genetic and phenotypic follow-up of patient populations with the same genetic variant is possible.

The variant TTR(NM_000371.4):c.14G>A, (p.Arg5His) was initially classified as a variant of unknown clinical significance, although it has not been reported to date as a variant directly associated with ATTRv. According to the genetic variant classification recommendations of the American College of Medical Genetics and Genomics (ACMG)[7], the *TTR* variant c.14G>A, p.Arg5His is classified as probably benign (categories of evidence BS2, BP4, PP2). The patient in whom the p.Arg5His variant was found suffered from type II diabetes and was later diagnosed with diabetic sensorimotor polyneuropathy. The available data lead to the probable lack of amyloidogenic function of the *TTR* variant c.14G>A, p.Arg5His, but its full clinical significance needs to be further verified by functional, clinical and population studies.

The results of the genetic screening in patients with a suspected diagnosis of ATTRv confirmed exons 2 and 3 of the *TTR* gene as a hotspot in Bulgaria for the accumulation of amyloidogenic variants leading to hereditary transthyretin amyloidosis. The diagnostic algorithm for genetic testing in patients with suspected diagnosis transthyretin amyloidosis was verified and summarized in the scheme shown in Figure 3.

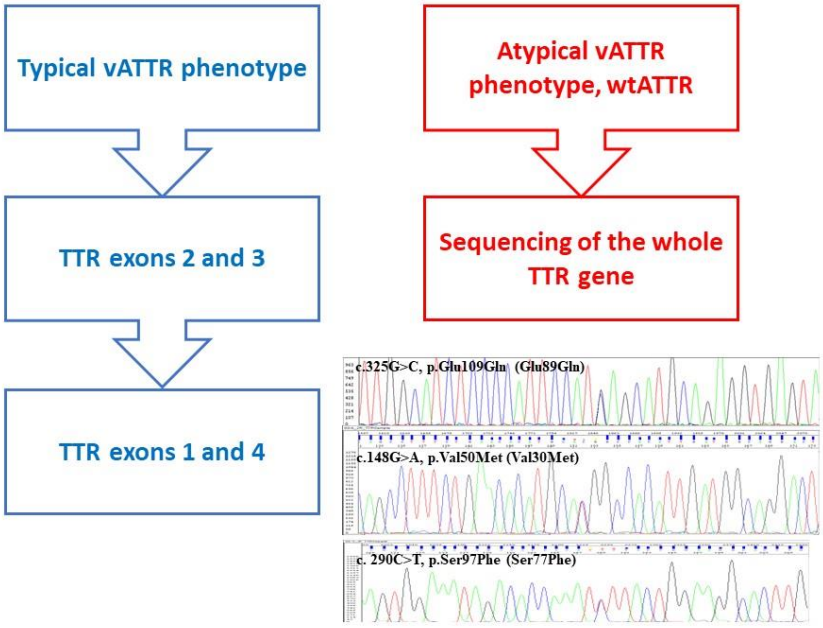

Figure 3. Algorithm for genetic testing in patients with suspected diagnosis transthyretin amyloidosis in Bulgaria. Sequencing electrophoregram in carriers of the three most common pathogenic variants in the *TTR* gene for Bulgaria, which are located in exons 2 and 3.

The information on the frequency of pathogenic *TTR* variants found in Bulgaria was updated (Table 5). At the moment, the Bulgarian ATTRv gene pool was supplemented with a new amyloidogenic variant Glu54Leu. Despite the small size of the *TTR* gene and the relatively well-studied mechanism of pathogenesis, new genetic variants associated with the development of ATTRv are still being discovered, as well as variants with difficult clinical or phenotypic interpretation. In addition, the results of screening for asymptomatic carriers in affected families proved the importance of these studies in terms of early diagnosis and treatment of ATTRv.

Table 5. Summary of the frequency of *TTR* pathogenic variants detected so far in Bulgaria.

***TTR* pathogenic variant (NM_000371.3, GRCh37)**	**Number of carriers in Bulgaria**	**Frequency among carriers**
c.325G>C; p.Glu109Gln (Glu89Gln)	278	77.22%
c.148G>A, p.Val50Met, (Val30Met)	39	10.83%
c.290C>T, p.Ser97Phe (Ser77Phe)	29	8.06%
Compound heterozygote c.148G>A, p.Val50Met, (Val30Met) + c.325G>C; p.Glu109Gln (Glu89Gln)	1	0.28%
c.200G>A; p.Gly67Glu (Gly47Glu)	7	1.94%
c.214T>C , p.Ser72Pro (Ser52Pro)	2	0.56%
c.220_221delGAinsCT, p.Glu74Leu (Glu54Leu)	4	1.11%
Total number of carriers	**360**	

3.1.2. Newborn screening to determine carrier frequency of the *TTR* variant Gly47Glu

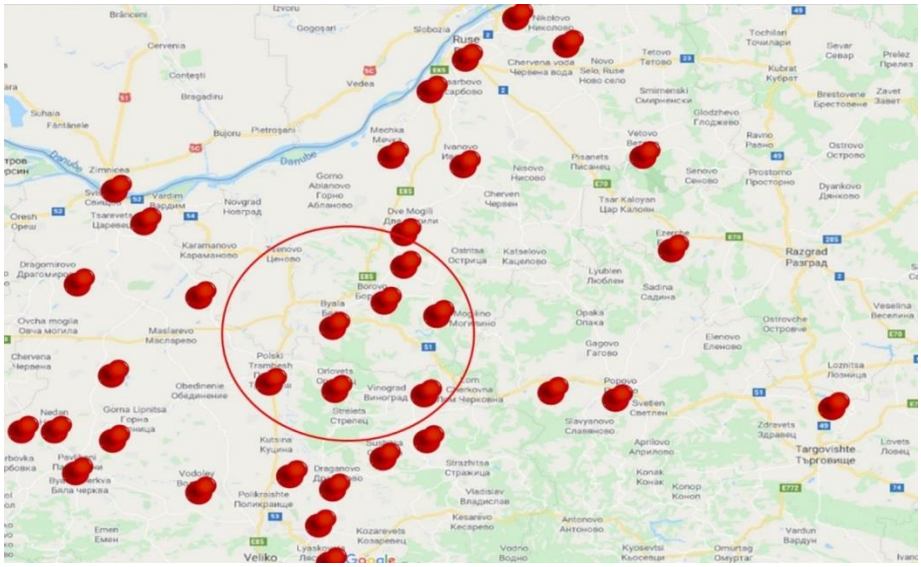

Figure 4. Geographical distribution of DBS tested for the Gly47Glu variant. The two proven ATTRv families with this variant are from the area of the city of Polski Trumbesh with a current population of 13,000 (shown in red circle).

Since the hypothesis for the presence of an endemic region for the *TTR* variant Gly47Glu was accepted as possible, we proceeded to investigate the frequency of carriership of this genetic variant among the Roma ethnic population from the region of origin of the two proven Gly47Glu families. For this purpose, 100 DBS samples of newborns from the region of the city of Polski Trumbesh and the city of Ruse were selected (Figure 4). The results in all tested samples were negative for pathogenic variants in exon 2 of the *TTR* gene. Besides Gly47Glu, the most frequent *TTR* pathogenic variant Val30Met for this exon was also not detected. Therefore, the hypothesis of Gly47Glu endemicity in the studied region of Bulgaria was rejected.

The results of this screening study indicated that the Gly47Glu variant most likely arose as a result of a recent single sporadic event, and the two affected families detected were most likely closely related. There are another 4 amyloidogenic substitutions in codon 47 of the *TTR* gene coding sequence, again making this locus a mutational hotspot[5]. The Gly47Glu variant has so far been reported in isolated cases in patients from Italy, Germany, Turkey, etc., with no evidence of endemicity or founder effect in these populations[8(p47),9(p47),10].

3.2. Analysis of regulatory genetic polymorphisms with potential effect on the clinical presentation of the disease

Table 6. Allelic frequency of the investigated genetic polymorphisms in ATTRv patients with Glu89Gln in Bulgaria. Frequencies were compared with general data for the European population.

Polymorphism	Europeans gnomAD v2.1.1				Glu89Gln Bulgaria			
	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
rs875119	T:	0.949	C:	0.051	T:	0.984	C:	0.016
rs3764478	G:	0.888	T:	0.112	G:	0.863	T:	0.137
rs1800458	G:	0.926	A:	0.074	G:	0.927	A:	0.073
rs72922947	G:	0.986	A:	0.014	G:	1.000	A:	0.000
rs7235277	G:	0.673	C:	0.327	G:	0.642	C:	0.358
rs62093482	C:	0.972	T:	0.028	C:	0.984	T:	0.016
rs1791228	C:	0.536	T:	0.464	C:	0.540	T:	0.460
rs4799586	T:	0.955	C:	0.045	T:	0.984	C:	0.016
rs1667245	C:	0.999	G:	0.001	C:	1.000	G:	0.000
rs35197841	TAGTAG:	0.999	TAG:	0.001	TAGTAG:	0.978	TAG:	0.022
rs76184052	T:	1.000	C:	0.000	T:	1.000	C:	0.000
rs72922938	C:	0.983	T:	0.017	C:	1.000	T:	0.000
rs723744	G:	0.671	T:	0.329	G:	0.511	T:	0.489
rs1080093	C:	0.625	G:	0.375	C:	0.556	G:	0.444

For the analysis of genetic polymorphisms with a potential effect on the clinical manifestation of hereditary transthyretin amyloidosis in the Bulgarian population, 14 single nucleotide polymorphisms were selected, which were genotyped in 124 DNA samples of patients carrying the *TTR* variant Glu89Gln. Data from direct sequencing of these genetic variants were used to calculate allelic frequencies in the selected Glu89Gln cohort. The results were compared with the available information on the frequency of genetic variants in the European population

from the GnomAD v.2.1.1[11] database (Table 6). The comparison shows that the frequency of these variants in the studied patient group largely coincides with the general data for the European population.

A correlation analysis was then performed regarding the genotype of the patients with respect to the studied polymorphisms and the clinical presentation of ATTRv. Clinical presentation was analyzed with respect to two parameters – age at disease onset and initial system involvement.

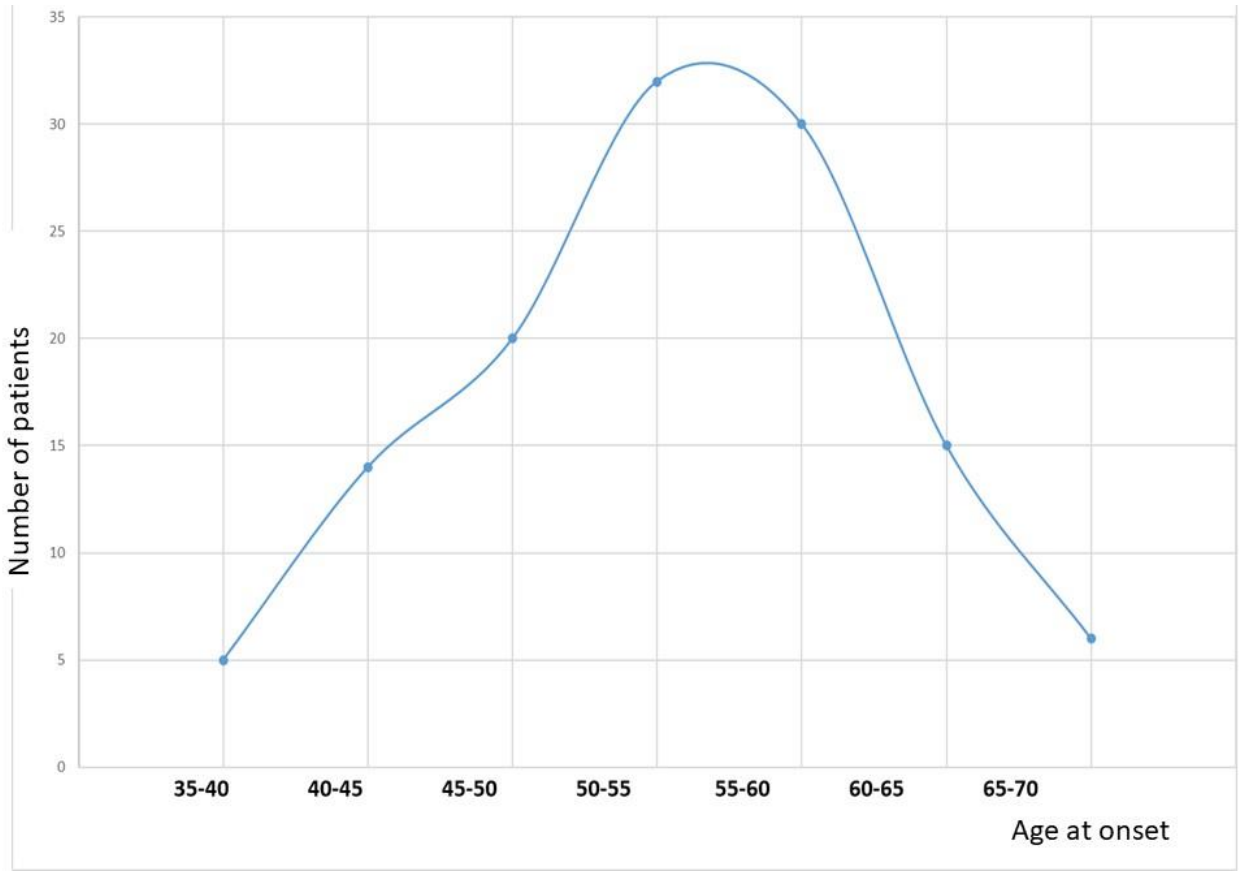

Figure 5. Distribution of ATTRv Glu89Gln patients by age at onset.

The performed statistical analysis aimed to investigate a possible disease-modifying effect of some genetic polymorphisms, the effect of which has already been studied in other ATTRv populations[12,13]. For this purpose, it was necessary to conduct stratification of the patient group according to the studied parameters (age at onset and initial system involvement). Therefore, before the statistical processing of the genetic results, a characterization of the studied group of patients was carried out in terms of the studied phenotypic characteristics. Initially, the distribution of patients by age at onset was assessed (Figure 5). For this purpose, the patients were divided into the following age at onset groups: 35-40 years, 40-45 years, 45-50 years, 50-55 years, 55-60 years, 60-65 years, 65-70 The results showed that more than 50% of patients fall into the range of age at onset 50-60 years of age, which determines the highest probability that a patient with Glu89Gln will present with their disease precisely in this age range. However, of great interest are the small number of patients falling in the extremes of the age curve of a normal distribution. According to literature data from studies mainly on Val30Met patients, the

age at onset of the disease is most often defined as early (under 40 years) and late (over 50 years)[14]. Due to the specificity of the age distribution observed in the Bulgarian Glu89Gln patients, for the purposes of the subsequent statistical analysis, the group was further divided into patients with early (under 45 years of age), intermediate (45-55 years of age) and late onset (over 55 years of age). The reported difference in age at onset versus patient gender was not considered in the present study.

Regarding the initial system involvement, patients were divided into groups, depending on whether the initial symptoms were related to the peripheral nervous system, heart, gastrointestinal tract, or mixed involvement (Figure 6). The most common initial symptoms are related to the development of polyneuropathy, while cardiac and gastrointestinal presentation of the disease occurs in a smaller percentage of Glu89Gln patients[15].

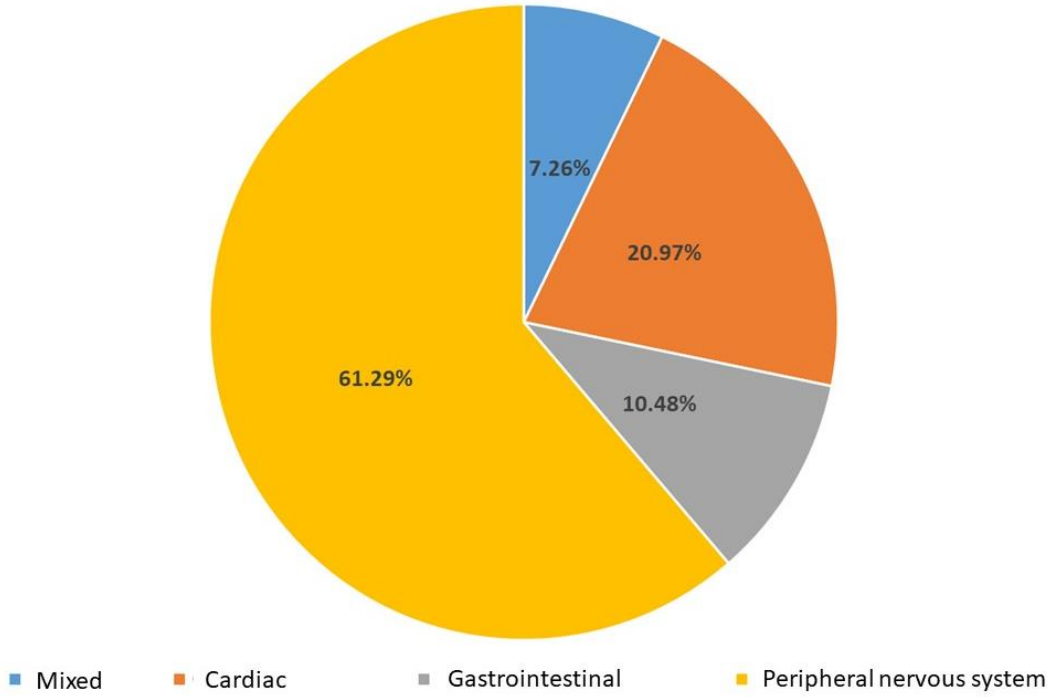

Figure 6. Initial system involvement in Glu89Gln ATTRv patients.

Table 7. Results of Chi-square test for correlation between studied genetic polymorphisms and age at onset and system involvement in Glu89Gln ATTRv patients. Marked in red are the results satisfying an alpha significance level of 0.05.

rs ID	**rs875119**	**rs3764478**	**rs1800458**	**rs7235277**	**rs62093482**
Age at onset (p-value)	0.268	0.399	0.405	0.223	0.268
Initial system involvement (p-value)	0.122	0.768	0.874	0.837	0.733
rs ID	**rs1791228**	**rs4799586**	**rs35197841**	**rs723744**	**rs1080093**
Age at onset (p-value)	**0.045**	0.268	0.464	0.398	0.809
Initial system involvement (p-value)	0.388	0.122	0.733	0.231	0.668

Correlation between a given allele of a genetic polymorphism and the clinical manifestation of ATTRv (age at disease onset and system involvement) was examined by Pearson's Chi-square test (Table 7). A statistically significant correlation was calculated only for the rs1791228 polymorphism with respect to age at disease onset at an alpha level of significance of 0.05.

The hypothesis that can be proposed based on these results is that patients who are heterozygous carriers of the T allele of the rs1791228 variant are more likely to develop the disease at a later age compared to homozygous carriers of the C allele (Figure 7). No homozygous carriers of the T allele were detected.

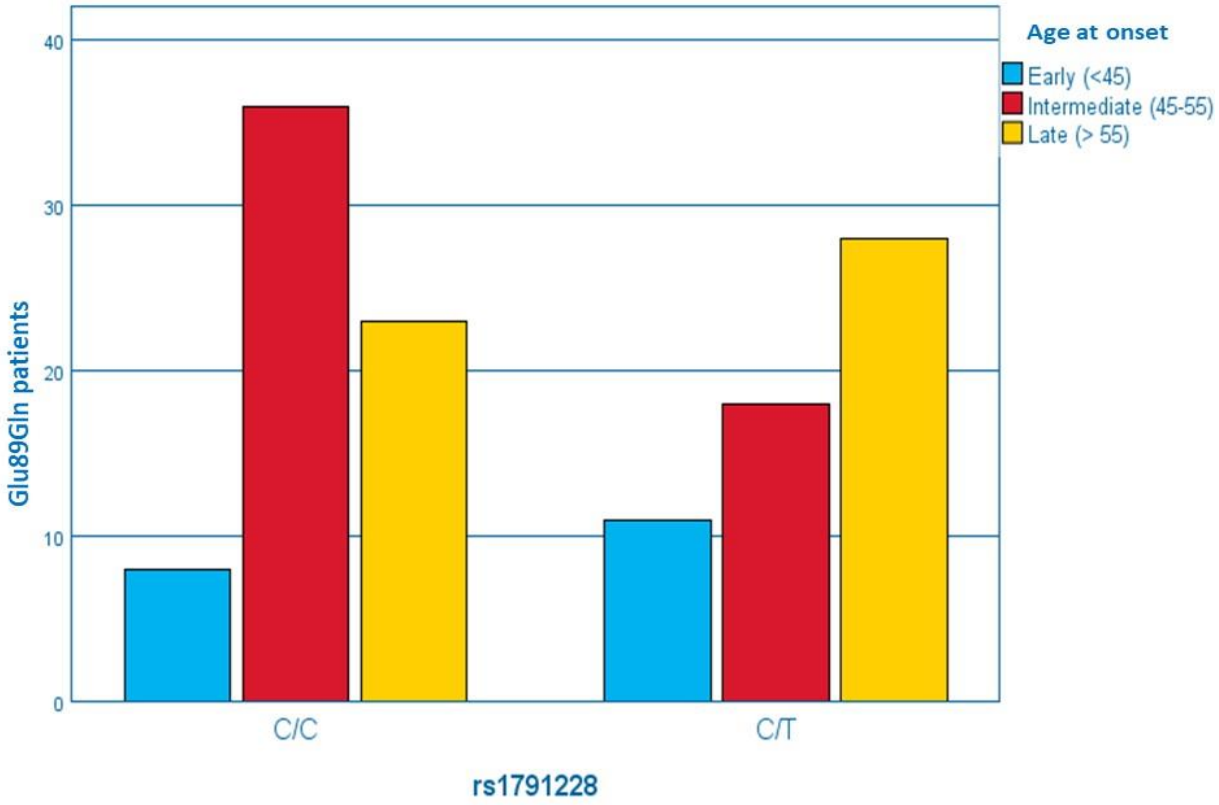

Figure 7. Distribution of Glu89Gln patients by age at disease onset and rs1791228 genotype.

Due to the proposed hypothesis, we moved on to a more detailed examination of patients carrying rs1791228. Detailed analysis showed that the mean age at disease onset in carriers of the T allele (54.50) was about two years higher than those carrying the homozygous C/C genotype (52.17). Figure 8 shows that the group of homozygous carriers of the more common C allele can reach a much earlier onset at onset than the group with the C/T genotype. However, over half of the individuals in both genotype groups showed an age of onset in the range of 48 – 57 (CC) and 48 – 59 (CT) years of age. Examination of a larger number of patients in the early-onset group (under 45 years of age) would probably have made adjustments to the calculated statistical probability. Although the statistical test showed no correlation between the rs1791228 genotype and the initial system involvement, an additional analysis of the patients was also performed regarding this hypothesis (Figure 9). There was a comparable increase in the percentage of heterozygous C/T patients in the primary cardiac and primary mixed involvement groups.

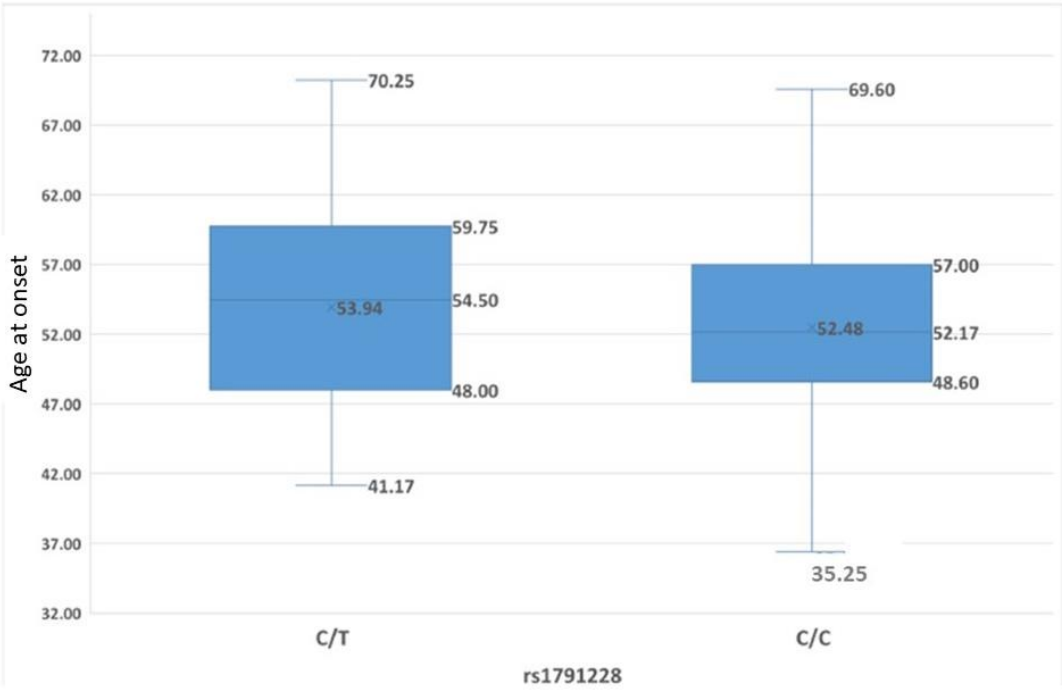

Figure 8. Numerical distribution and mean age at onset in the two rs1791228 genotype groups – C/C and C/T. The minimum age at onset in heterozygous carriers was 41.17, while the minimum age in homozygous C/C individuals was 35.25. However, the mean age of the two groups differed by only two years.

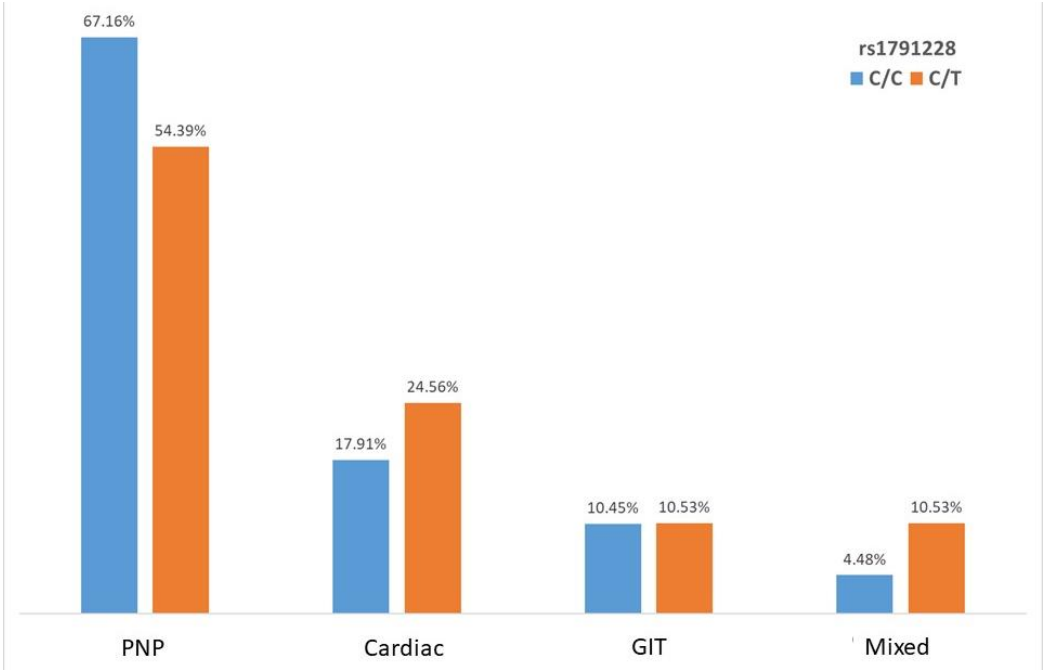

Figure 9. Numerical distribution and initial system involvement in the two rs1791228 genotype groups – C/C and C/T. Heterozygous carriers of the T allele show a tendency towards initial cardiac or mixed involvement compared to homozygous carriers of the more common C allele. PNP - polyneuropathy, GIT - gastrointestinal tract.

The rs1791228 polymorphism has already been reported in correlation with age at disease onset in Portuguese patients with Val30Met[16]. It is a non-coding variant located downstream of the *TTR* gene, which is a possible transcriptional regulator. In silico analyzes show a possible splicing role of this genetic variant by activating a branch point that is inactive in the wild-type sequence[16]. Another proposed regulatory mechanism of rs1791228 is the disruption of a binding site for the transcription factor TEAD1 (Figure 10)[17,18]. TEAD1 has a role in the regulation of tissue-specific gene expression in cardiomyocytes through transcriptional activation of genes for cardiac troponin T, skeletal muscle actin, and myosin heavy chains[19(p1)]. The studied genetic variant rs1791228 has been reported in a possible haplotype with the pathogenic variant Val122Ile, which is characterized by predominantly cardiac involvement[17]. These data match the observed correlations in the Bulgarian Glu89Gln cohort, but the statistical or mathematical correlation is not sufficient to absolutely determine the clinical phenotype of ATTRv. Most probably a number of genetic markers with different functional effect and significance, in combination with epigenetic factors, most likely underly the observed clinical heterogeneity, which remains to be explored in future studies.

Figure 10. Transcription factor binding sites potentially disrupted by the T allele of the rs1791228 polymorphism. Source: RegulomeDB v2.0.3[20].

3.3. Transthyretin gene expression analysis

The proposed methodology for gene expression analysis was initially tested on an RNA sample extracted from liver tissue with an expected high expression of transthyretin. The results from the polymerase chain reaction optimization showed amplification of a cDNA fragment of the predicted length of 290 base pairs (Figure 11), which by direct Sanger sequencing was

confirmed to be a transthyretin mRNA transcript. Subsequent analysis on the noninvasive samples was performed using the optimized PCR protocol with a primer hybridization temperature of 62°C.

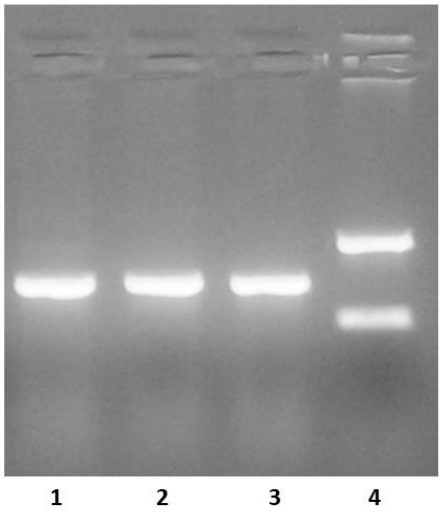

Figure 11. Agarose electrophoresis of cDNA fragments of the transthyretin transcript from PCR optimization on a control RNA sample extracted from liver. Lanes 1 to 3 represent cDNA fragments amplified with primers that complement the ends of adjacent TTR exons by different primer hybridization temperature: 1 - 58°C, 2 - 60°C, 3 - 62°C. Lane 4 contains two control DNA fragments of 175 and 421 base pairs used for relative sizing.

Transcriptional analysis of patients' blood and urine samples was further performed. Samples from 19 Glu89Gln patients from 6 families whose pedigrees are shown in Figure 12 were analyzed. Analysis of the RNA TTR sequencing results showed a mixed transcriptional profile of the mutant and wild-type allele in different ratios (Figure 13). Comparative analysis of the profiles obtained from blood plasma and urine showed that a similar proportion of expression of the mutant allele was observed in both types of samples (about 40%). Fragment analysis of allele-specific cDNA fragments was used to calculate the relative quantitative ratio between the two allelic variants. For normalization, the area of the wild-type allele was used as 1 unit in each sample. The results showed a variation of the amount of mutant transcript in the range of 0.11 – 1.14 units, which correlates with the original data from the sequencing electrophoregrams.

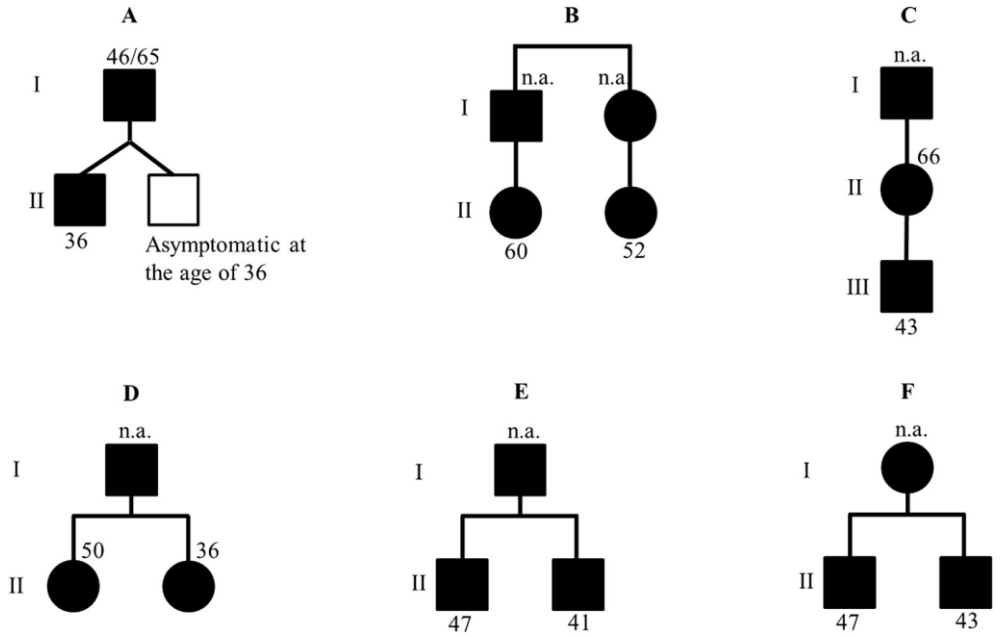

Figure 12. Pedigrees of the Glu89Gln ATTRv families involved in the transthyretin expression study. Roman numerals indicate the generations, and Arabic numerals indicate the age at onset of the disease. "n.a." denotes the lack of information available regarding the age at disease onset in some of the patients.

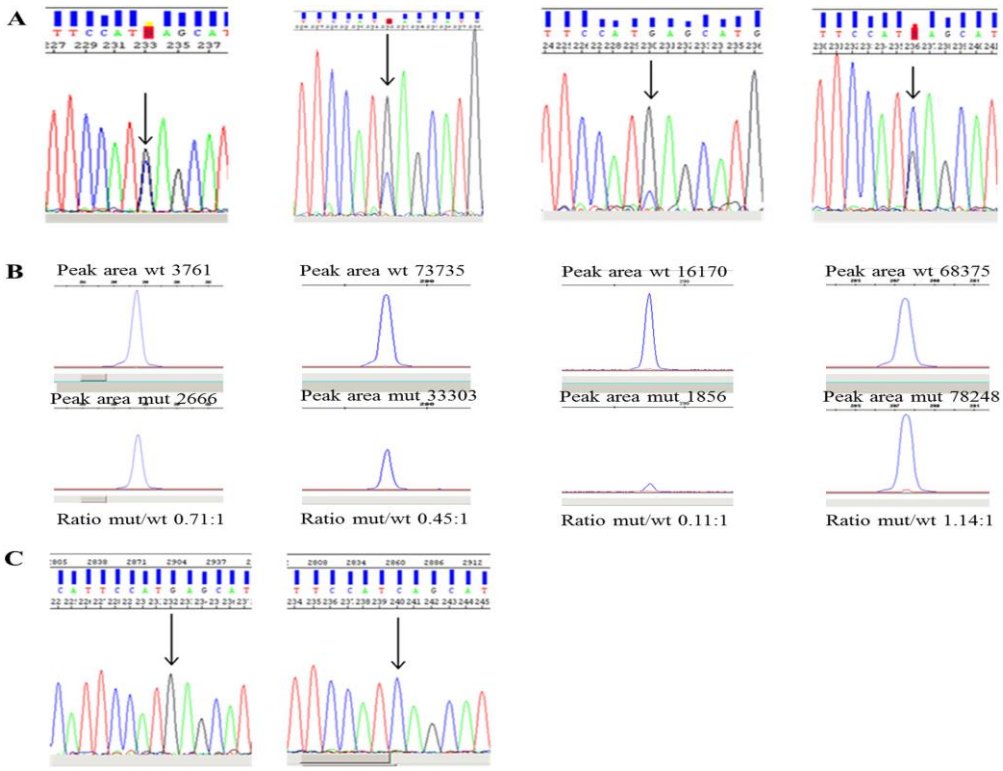

Figure 13. Transthyretin expression in some of the tested samples. A. Sequencing electrophoregrams from direct Sanger sequencing of the cDNA fragments. B. Relative quantitative analysis of the wild-type allele (wt) and the mutant (mut) allele in the same test samples. C. Samples showing 100% monoallelic expression.

The presence of monoallelic transcriptional profiles in both blood and urine samples was surprising. Summarized results showed an 87% monoallelic profile in the blood plasma samples versus 13% biallelic expression. For the urine samples, the results showed 58% monoallelic versus 42% biallelic expression (Figure 14). A tissue-specific type of transthyretin expression (monoallelic expression in urine vs. biallelic expression in plasma and vice versa) was reported in some patients. Blood plasma samples showed a higher percentage of monoallelic expression of the mutant allele than urine samples.

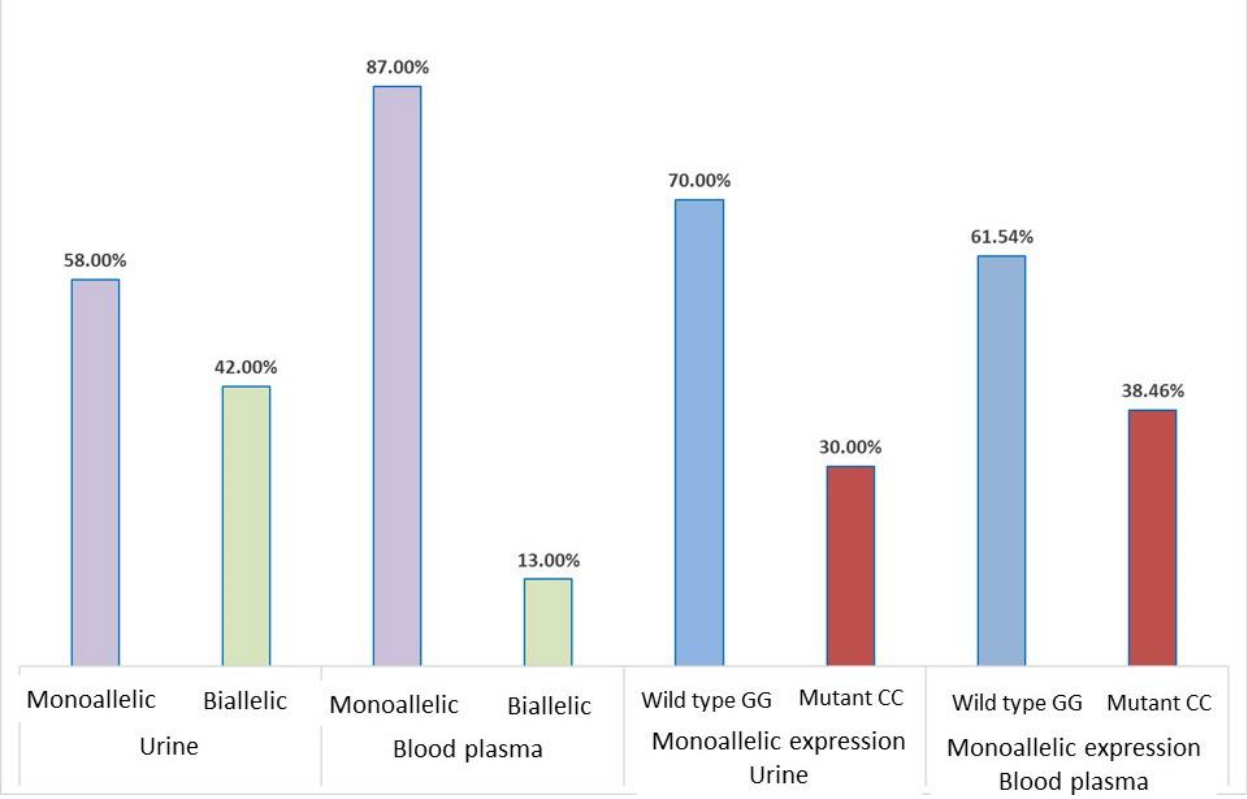

Figure 14. Results of the analysis of allele-specific transthyretin gene expression in the studied samples.

Of great interest were the results of the family with two monozygotic twins carrying Glu89Gln. In this family, the difference in the age at onset is more than 10 years. The father was operated for carpal tunnel syndrome initially on the right hand at the age of 46 and then on the left hand at the age of 50. At the age of 58, he experienced an ischemic stroke, and at the age of 64, he was diagnosed with left ventricular heart hypertrophy. In contrast, one of his two sons showed symptoms of transthyretin amyloidosis at age 35 (11 years earlier). His symptoms started again with bilateral carpal tunnel syndrome and subsequently developed a polyneuropathic syndrome. At the age of 42, he was also diagnosed with restrictive cardiomyopathy. His twin brother was asymptomatic until the age of 46, when mild neurological complaints began in the upper extremities. Both brothers were sampled twice for transcriptional analysis within a year. In the patient with an earlier onset of the disease, the presence of a biallelic profile in urine and a wild-type monoallelic profile in blood was observed at the initial examination of transthyretin expression, while in the patient with a later onset both samples (blood and urine) were initially examined. show monoallelic expression of the wild-type transcript. Re-examination of both brothers showed the presence of expression of the mutant allele in both brothers. Based on these

results, we propose a hypothetical expression mechanism that involves age-dependent allele-specific transthyretin expression: predominant expression of the wild-type allele at an earlier age, which gradually switches to expression of the mutant allele with advancing age (Figure 15).

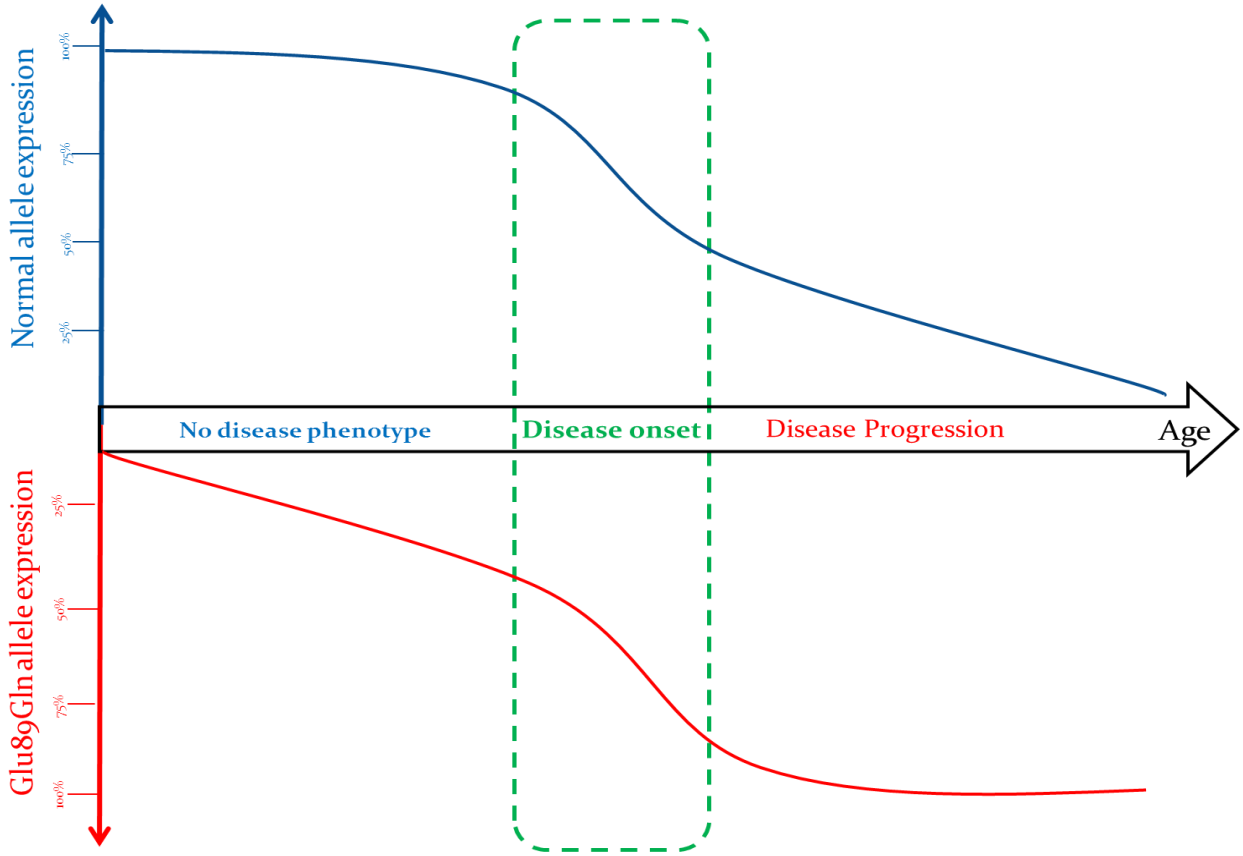

Figure 15. Hypothetical model of age-dependent allele-specific expression of the *TTR* gene in patients with Glu89Gln.

Monoallelic expression could be explained by dependence on the sex of the transmitting parent, i.e. through the presence of gene imprinting. Another known mechanism is random monoallelic expression, which occurs as a result of random inactivation of one homologous allele in the cell[21]. Due to the presence of biallelic expression and the small number of patients studied, no final conclusion can be drawn regarding the mechanism of gene expression of the transthyretin gene.

The presence of interfamilial and intrafamilial differences in the clinical manifestation of various autosomal dominant genetic diseases, including hereditary transthyretin amyloidosis, has been the subject of investigation regarding the molecular mechanisms behind the observed phenotype[22]. There are numerous studies on the presence of epigenetic molecular changes associated with aging, including methylation of regulatory regions of the genome, histone modifications, etc., which in turn can lead to the use of alternative promoters and spatial reorganization of chromatin over time[23]. These so-called epigenetic "clocks"[24] could underlie the observed differences in the transcriptional profiles of the studied Glu89Gln ATTRv patients, in which switching between mono- and bi-allelic transthyretin expression could hypothetically be relevant to disease onset and progression.

The results from our patients showed the presence of dynamically changing monoallelic and biallelic expression, leading to a more complex mechanism of tissue-specific gene transcription that is likely determined by a set of genetic and epigenetic factors. The study of monozygotic twins has been extremely valuable for the analysis of similar biological processes[25]. Despite the proposed hypothetical model of age-dependent allele-specific transthyretin expression, the relationship between the specific allelic type of transthyretin expression and the presence of a specific disease phenotype at present remains unclear. It is probably necessary to investigate a larger cohort of patients from the asymptomatic carrier stage to the appearance of the first clinical complaints with subsequent disease progression to be able to examine the expression hypothesis more precisely. However, the observed results lay a promising basis for clarifying the clinical manifestation of transthyretin amyloidosis in the context of the most common *TTR* genetic variant Glu89Gln which is endemic in Bulgaria.

3.4. Haplotype analysis to determine the founder effect

3.4.1. Microsatellite fragment analysis

Microsatellite markers in the region of the *TTR* gene were successfully amplified and analyzed by fragment analysis in all 205 examined DNA samples of families carrying the four most common pathogenic variants in Bulgaria, as well as in the control DNA samples. After obtaining data on the alleles of the markers in each sample, an analysis of the degree of heterozygosity in the study population was performed (Figure 16). The results showed that for five of the six markers, over half of the individuals carry a heterozygous genotype. This means that the selected markers can be used for statistical analysis and are suitable for tracing the inheritance of *TTR* variants in our population.

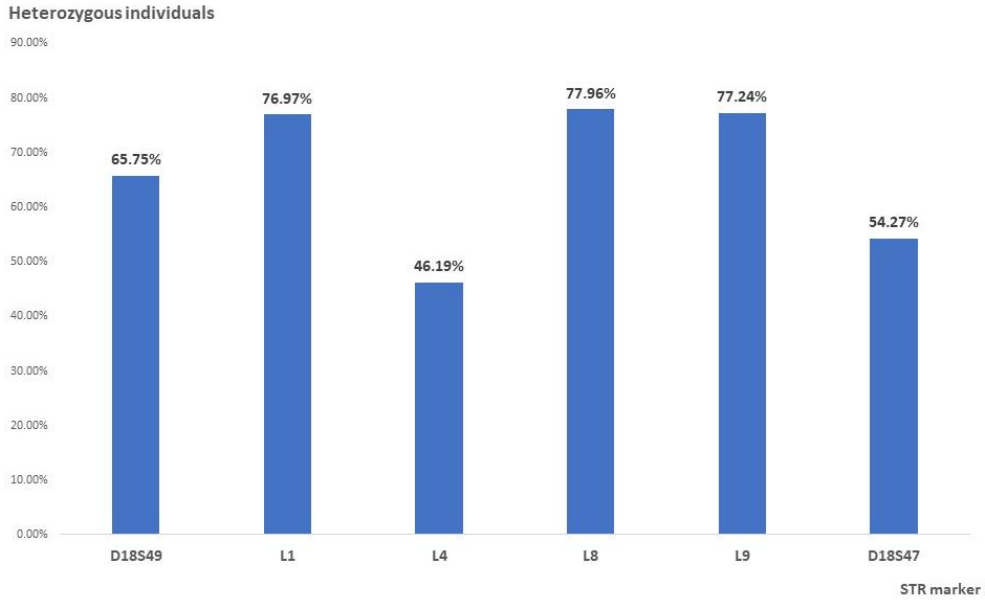

Figure 16. Percent heterozygosity of the selected microsatellite markers in the studied population. The heterozygosity of marker L4 was only slightly lower than 50% (46.19%), therefore it was decided not to exclude this marker from the study**.**

The allelic frequencies of microsatellite markers in the subgroups of ATTRv patients carrying each of the four *TTR* pathogenic variants (Glu89Gln, Val30Met, Ser77Phe, Gly47Glu) were analyzed. The most frequent alleles were called alleles of the hypothetical founder haplotype and their frequencies were compared in the subgroups of the normal relatives and the control group (Table 8). The results showed that these alleles were found predominantly in the group of ATTRv patients. The presence of a founder effect usually leads to linkage disequilibrium between the alleles of certain loci inherited from the founder individual. This effect would explain the difference between the observed allelic frequencies and the frequencies that would be expected in the presence of random allelic association (Hardy-Weinberg equilibrium[26]).

Table 8. Frequencies of the hypothetical founder haplotype alleles for the *TTR* variants Glu89Gln, Val30Met, Ser77Phe and Gly47Glu.

Glu89Gln	***D18S49 115 bp***	***L1 245bp***	***L4 267bp***	***L8 292bp***	***L9 324bp***	***D18S47 202bp***
Glu89Gln patients (N=36)	**0.50**	**0.32**	**0.72**	**0.50**	**0.40**	**0.39**
Healthy relatives (N=31)	0.08	0.00	0.48	0.25	0.21	0.29
Control group (N=40)	0.01	0.03	0.00	0.19	0.08	0.01
Val30Met	***D18S49 104 bp***	***L1 242bp***	***L4 262bp***	***L8 291bp***	***L9 323bp***	***D18S47 198bp***
Val30Met patients (N=26)	**0.35**	**0.48**	**0.58**	**0.46**	**0.40**	**0.56**
Healthy relatives (N=15)	0.30	0.10	0.57	0.43	0.27	0.67
Control group (N=40)	0.10	0.10	0.34	0.34	0.14	0.46
Ser77Phe	***D18S49 107bp***	***L1 244bp***	***L4 264bp***	***L8 291bp***	***L9 323bp***	***D18S47 198 bp***
Ser77Phe patients (N=25)	**0.52**	**0.28**	**0.88**	**0.56**	**0.48**	**0.78**
Healthy relatives (N=18)	0.08	0.17	0.78	0.47	0.33	0.50
Control group (N=40)	0.35	0.03	0.46	0.34	0.14	0.46
Gly47Glu	***D18S49 104bp***	***L1 242bp***	***L4 262bp***	***L8 295bp***	***L9 307bp***	***D18S47 202 bp***
Gly47Glu patients (N=6)	**0.67**	**0.58**	**0.58**	**0.50**	**0.25**	**0.67**
Healthy relatives (N=8)	0.44	0.13	0.44	0.06	0.06	0.13
Control group (N=40)	0.10	0.10	0.34	0.06	0.00	0.43

3.4.2. Haplotype reconstruction

Based on the results of the fragment analysis, a theoretical reconstruction of the haplotype of each individual in the subgroups of patients, healthy relatives and controls was performed using the ELB algorithm of the Arlequin v.3.01 software. The most frequent mathematically reconstructed haplotypes for the ATTRv patients were shown to consist of the

alleles of the hypothetical founder haplotype, and these haplotypes were again mostly found only in the ATTRv patient group. The results are summarized in Table 9.

Table 9. Most frequent haplotypes for the studied TTR variants Glu89Gln, Val30Met, Ser77Phe and Gly47Glu in the studied ATTRv families.

D18S49	L1	L4	*TTR pathogenic variant*	L8	L9	D18S47	*ATTRv patients*	*Healthy relatives*
115	245	267	***Glu89Gln***	292	324	202	***37%***	***0%***
104	242	262	***Val30Met***	291	323	198	***27%***	***0%***
107	244	264	***Ser77Phe***	291	323	198	***23%***	***0%***
104	242	262	***Gly47Glu***	295	307	202	***21%***	***7%***

In Figures 17, 18 and 19 are selected pedigrees of some of the studied families to demonstrate the transmission of the reconstructed haplotypes in the generation and the linkage to the particular *TTR* pathogenic variant. Of particular interest is the family in which the compound heterozygous Glu89Gln / Val30Met carrier was found. It is noted that the Val30Met-linked haplotype in this family has undergone a decay compared to the theoretical founder haplotype determined from all Val30Met carriers.

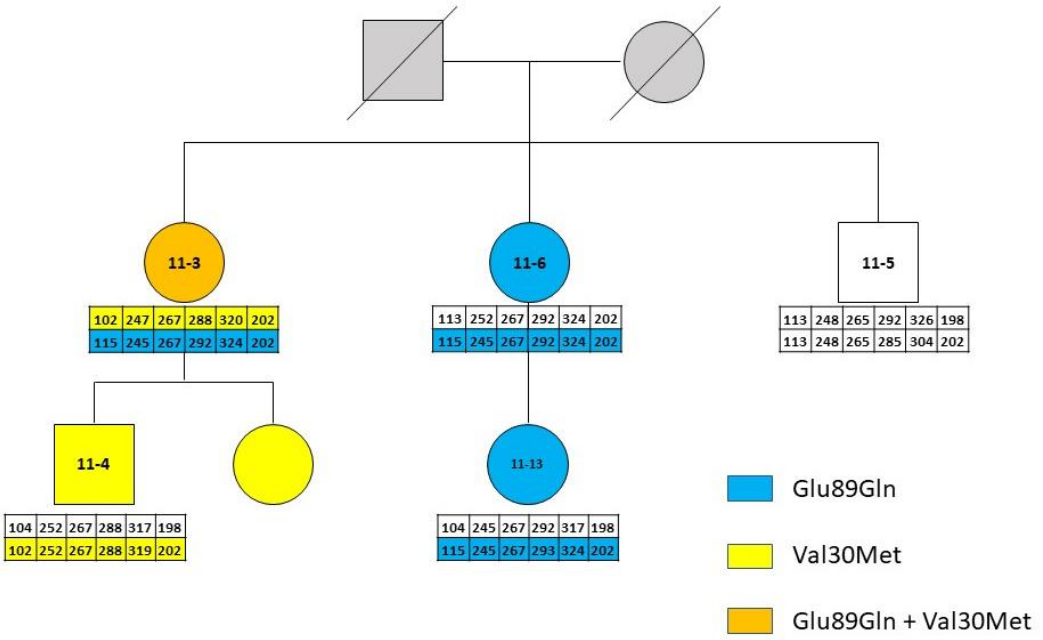

Figure 17. Pedigree and haplotype inheritance in a family carrying Glu89Gln and Val30Met.

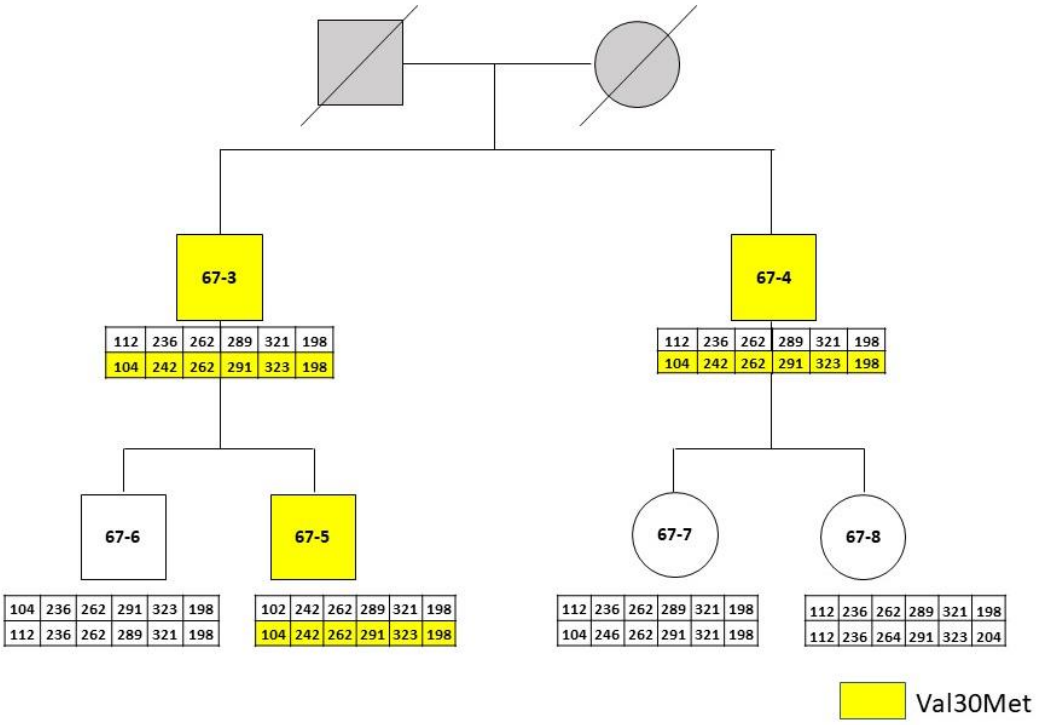

Figure 18. Pedigree and haplotype inheritance in a Val30Met carrier family.

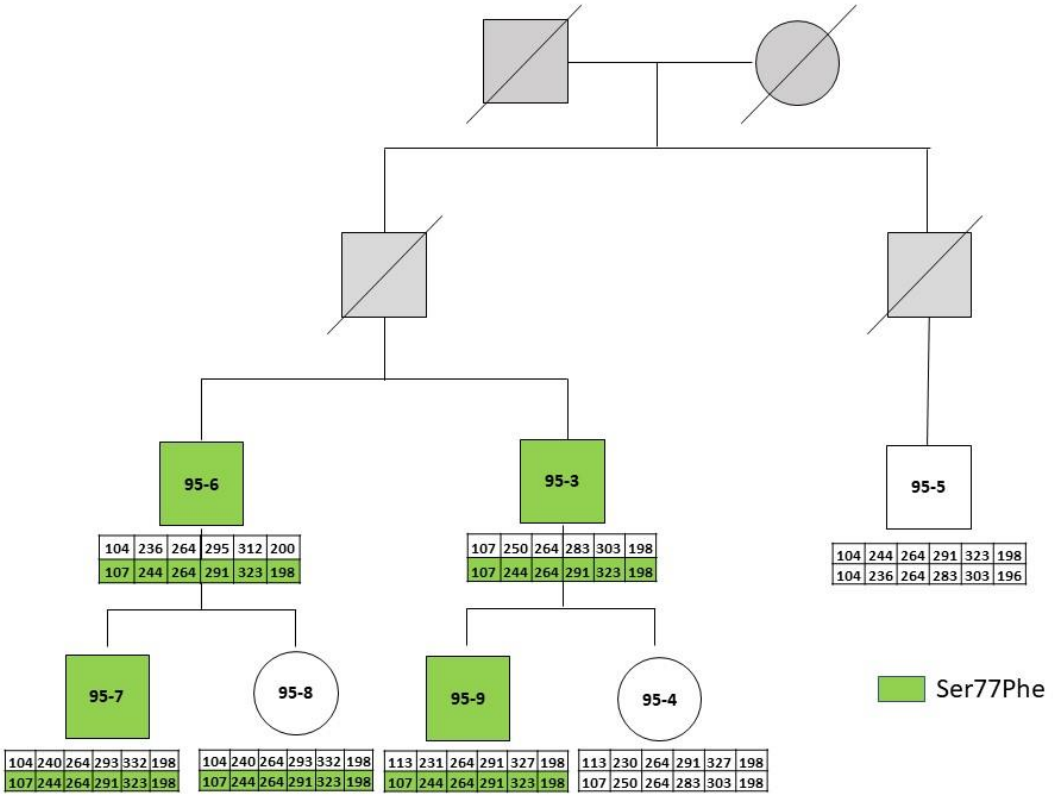

Figure 19. Pedigree and haplotype inheritance in a Ser77Phe carrier family.

3.4.3. Statistical evaluation

Based on the data on the allele frequencies of the microsatellite markers among the subgroups of ATTRv patients, healthy relatives and controls, the hypothesis of the presence of a founder effect for each of the four *TTR* pathogenic variants was statistically evaluated. The conducted Chi-square test showed the presence of an association between the tested variables (TTR status and carriage of the alleles of the hypothetical founder). The respective p-values were considered at an alpha significance level of 0.05 (Table 10).

Table 10. Results of the performed Pearson's Chi-square test. The p-values at an alpha significance level of 0.05 are shown.

TTR pathogenic variant	***D18S49*** *Founder allele*	***L1*** *Founder allele*	***L4*** *Founder allele*	***L8*** *Founder allele*	***L9*** *Founder allele*	***D18S47*** *Founder allele*
Glu89Gln	<0.0001	<0.0001	0.0050	0.0040	0.0160	<0.0001
Val30Met	0.0034	<0.0001	<0.0001	<0.0001	<0.0001	0.0010
Ser77Phe	<0.0001	0.0010	0.0110	<0.0001	<0.0001	<0.0001
Gly47Glu	0.0170	<0.0001	0.0170	<0.0001	0.0180	<0.0001

3.4.4. Determining "age of mutation" - the age of the most recent common ancestor

Based on the reconstruction of the haplotypes carried out in the course of the present work, a mathematical simulation was carried out to determine the theoretical age of the most recent common ancestor for each of the examined pathogenic variants in the *TTR* gene. Data for the decay of the hypothetical founder haplotype were analyzed using DMLE+ v.2.3 software. The recombination frequency between the studied loci was taken into account, based on the data from the relative localization of the studied microsatellite markers to each of the pathogenic *TTR* variants. The results presented in Table 11 are at a 95% confidence interval and with the approximation that one generation equals 25 years[27].

Table 11. Results from the mathematical determination of the age of the most recent common ancestor presented in number of generations. One generation equals 25 years.

TTR pathogenic	***Mean age of the most recent common ancestor***	***Age with 95% confidence interval***
Glu89Gln	**45**	**38-52**
Val30Met	**47**	**36-58**
Ser77Phe	**43**	**34-52**
Gly47Glu	**32**	**24-40**

The conducted molecular-genetic and statistical studies show with a high degree of probability the presence of a founder effect for the studied pathogenic variants in the *TTR* gene Glu89Gln, Val30Met, Ser77Phe and Gly47Glu. Forces, affecting populations that can lead to

loss of genetic diversity and the presence of a founder effect for a particular genetic defect include the cumulative effect of migrations, disasters, geographic or cultural isolation of a population[28]. In addition to the so-called genetic drift, ATTRv is a late-onset disease that does not allow the forces of natural selection to influence the elimination of pathogenic *TTR* variants from the population. Regarding Glu89Gln, the preliminary data on the existence of a clear endemic region for this genetic variant in southern Bulgaria was strongly supported by the results of the haplotype analysis. This made it clear that all Glu89Gln patients most likely descended from a common ancestor who introduced this variant into our population theoretically about 45 generations ago. The Glu89Gln variant is found mainly in Sicily, Sardinia and the European part of Turkey[29]. This geographic distribution in the context of past migration events in the Balkan and Mediterranean region would explain the data for founder effect Glu89Gln in Bulgaria.

The Val30Met variant shows a more scattered geographical distribution, compared to the other ATTRv pathogenic variants in Bulgaria. The results of the haplotype analysis and the theoretical calculation of the age of the most recent common ancestor indicate that the hypothetical founder who could have introduced Val30Met into our population appeared earlier than the other "founders". Val30Met patients showed a greater degree of decay of the funder haplotype, which could correlate with a greater "mutation age". Val30Met has been reported in a number of endemic regions worldwide, including Portugal, Spain, Italy, Sweden and Japan. Similar population genetic studies have been performed in some of these countries, which made it possible to make a comparison between the Bulgarian Val30Met haplotype and published haplotype data from other populations[30–32]. The results are summarized in Table 12. Bulgarian Val30Met patients differ significantly from patients from other endemic regions. The reason for this could be the more distant genetic origin of the Bulgarian Val30Met founder. On the other hand, since the locus of this genetic variant is assumed to be a mutational hotspot, it is possible that Val30Met arose independently on our lands as a separate genetic event. In comparison, Brazilian and Portuguese patients with Val30Met have proven common ancestry[32].

Table 12. Comparison between Val30Met haplotypes from different endemic populations around the world. *For the Spanish, Swedish and Japanese patients, there are no data for markers D18S49 and D18S47.

Val30Met haplotype	**D18S49**	**L1**	**L4**	**L8**	**L9**	**D18S47**
Bulgaria	104	242	262	291	323	198
Italy	122	237	271	297	311	202
Portugal	113	237	271	291	311	202
Brazil	113	237	271	291	311	202
Spain	*	235	271	291	321	*
Japan	*	235/237	271	299/305	311/327	*
Sweden	*	241	271	303	329	*

Bulgarian patients with Val30Met show a mixed and relatively mild phenotype (there are cases with onset of the disease over 70 years of age), which makes it difficult to take a family history and can complicates the accurate diagnostic process and the study of the epidemiology of the disease. Based on several studies on Val30Met patients from Japan and Portugal, it can be

concluded that an earlier age at onset and a more severe clinical presentation are observed in patients known to originate from endemic regions. in contrast to so-called 'sporadic' cases without a family history of the disease[33,34]. It is likely that the later age of onset of ATTRv and the milder clinical presentation in some of the Val30Met patients are the reasons why some of these cases have a difficult to trace family history and are considered sporadic. On the other hand, there are two separate endemic regions for Val30Met in Sweden, where differences in age at onset are observed in relation to the region of origin[35].

Families with the Ser77Phe variant in Bulgaria originated from a clearly defined endemic region (Vakarel village) and were also shown to likely originate from a common ancestor. Allele frequencies of the hypothetical founder haplotype occur at high frequency in Ser77Phe carriers, and this variant most likely arose in the population more recently than Glu89Gln and Val30Met. The Ser77Phe variant has also been reported in the French population[36]. In addition, another pathogenic amyloidogenic variant Ser77Tyr[37] has been described at the same gene position. It is likely that this locus also represents a hot spot for the accumulation of variants in the *TTR* gene.

Analysis of the two families in which the Gly47Glu variant was found indicated that they most likely descended from a common ancestor. However, the absence of other affected Gly47Glu families and the negative screening results in newborns from the region of origin of the two Gly47Glu families are evidence that the hypothesis of an ATTRv endemic region to the Roma population should rather be rejected. The significantly more aggressive nature of this pathogenic variant, which is characterized by the onset of the disease around 20-30 years of age, could be the reason for the low frequency in Bulgaria, which would potentially mean that this mutation will disappear from the population over time.

The origin of ATTRv patients affects the expected disease manifestation. The hypothesis of the presence of genetic and epigenetic factors influencing the heterogeneity of the clinical manifestation of the disease should be investigated in the context of different subgroups of patients who are carriers of the same *TTR* variant[31,38,39]. Future comparative studies between ATTRv populations carrying non-Val30Met pathogenic variants of the *TTR* gene would give us a more detailed picture of the epidemiology, prevalence and appearance of ATTRv worldwide.

3.5. Summary of results and conclusion

The studies carried out within the framework of this dissertation cover various aspects regarding the prevalence, clinical manifestation and molecular mechanisms of pathogenesis in patients with hereditary transthyretin amyloidosis in Bulgaria. The pathogenic *TTR* variant Glu89Gln remains the most common genetic defect, but a new variant for the country Glu54Leu was also discovered. The high proportion of positive asymptomatic carriers among the group of patients' relatives proves the need for screening in affected families in order to make a timely diagnosis. The negative screening results in newborns of Roma origin from the region of the city of Polski Trumbesh and Ruse rejected the hypothetical endemicity for the Gly47Glu variant, leaving the variants for which there are proven endemic regions in Bulgaria to be Glu89Gln, Val30Met and Ser77Phe.

The results of the analysis of potential genetic modifiers of ATTRv showed the presence of a statistical correlation between the age at onset and the presence of the T allele of the rs1791228 polymorphism. Analysis of the effect of this polymorphism on the initial system involvement in the studied cohort showed an increase in the proportion of patients carrying the T allele with initial cardiac and mixed involvement. The observed results largely coincide with the data reported in the literature, but the proposed molecular mechanisms are not yet sufficiently studied to make a reliable conclusion about the effect of this polymorphism on the development of ATTRv.

The results of transthyretin gene expression analysis showed a mixed mono- and biallelic transcriptional profile with different ratios between mutant and wild-type transthyretin, which could hypothetically have an effect on disease progression. A hypothetical mechanism for age-dependent allele-specific gene expression was proposed, but further studies are needed to clarify the relationship between the particular expression profile observed and the development of ATTRv.

The haplotype analysis of genetic microsatellite markers linked to the *TTR* gene confirmed mathematically and statistically the hypothesis of the presence of a founder effect for the studied groups of patients carrying the *TTR* variants Glu89Gln, Val30Met, Ser77Phe and Gly47Glu. The theoretical age of the most recent common ancestor for the four variant groups was calculated, finding that Val30Met was introduced most distantly into our population. The results of the conducted population-genetic studies showed the presence of a genetic profile in the Bulgarian ATTRv patients unique to the world, which is potential basis for the study of the still insufficiently well-studied differences in the phenotypic manifestation of the disease.

4. CONCLUSIONS

4.1. Exons 2 and 3 of the *TTR* gene remain a proven hotspot of accumulation of amyloidogenic variants causing hereditary transthyretin amyloidosis among Bulgarian patients. Therefore, the diagnostic algorithm for genetic testing should include initial testing of the hot region, with subsequent analysis of exons 1 and 4 in the presence of convincing clinical indications.

4.2. The two affected Gly47Glu families are most likely closely related and the presence of this variant is the result of a recent single mutational event. This rejects the hypothesis of the presence of an endemic region for Gly47Glu among the Roma population in Northern Bulgaria.

4.3. Age of disease onset in ATTRv Glu89Gln patients significantly statistically correlates with the genotype for the rs1791228 polymorphism, and this marker also showed a mathematical correlation with the presence of initial cardiac and mixed involvement. The data from the conducted studies coincide with the correlations reported in the literature, but further studies are needed to be able to make a reliable conclusion about the effect of the rs1791228 polymorphism on the manifestation of ATTRv.

4.4. The mixed transthyretin transcriptional profile (mono- and biallelic expression) of the examined blood plasma and urine samples of patients with Glu89Gln showed a partial correlation with the clinical presentation of the disease. The proposed hypothetical mechanism for age-dependent allele-specific gene expression involves an initial expression of the wild-type allele that gradually shifts to expression of the mutant allele.

4.5. Detailed analysis of transthyretin expression and its monitoring during the course of the disease could serve as a prognostic marker for the progression of ATTRv, but the mechanism of amyloidogenesis and tissue involvement includes a set of many other molecular factors (genetic and epigenetic).

4.6. The Bulgarian genetic pool is unique in terms of the origin and epidemiology of the most common amyloidogenic *TTR* variants for the Bulgarian population, Glu89Gln, Ser77Phe and Val30Met, for which the presence of a founder effect was reliably proved. The degree of geographic dispersal of the population of ATTRv patients from the endemic regions coincides with the decay of the hypothetical founder haplotype and the theoretically calculated “mutation age”.

5. CONTRIBUTIONS

Scientific and applied contributions:

5.1. The genetic pool in terms of amyloidogenic variants in the *TTR* gene was supplemented with a new variant for the country Glu54Leu, which is yet to be phenotypically characterized and tracked in the population.

5.2. The unique Bulgarian population-genetic profile compared to other ATTRv endemic populations around the world was proven, which provides a basis for conducting further research on the heterogeneity of the clinical manifestation of the disease.

Methodological contributions:

5.3. A method was developed for the qualitative and quantitative analysis of the transthyretin transcript in easy to obtain clinical material (blood plasma and urine). The method allows comparative analysis of allele-specific transthyretin expression, which can potentially serve as a marker for disease progression.

5.4. A founder effect methodology was successfully selected by applying the ELB algorithm for theoretical haplotype reconstruction, which allows the analysis of large data samples to conduct a range of population genetic and correlational studies.

6. SCIENTIFIC PUBLICATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION

6.1. PUBLICATIONS

1. Kirov A, Sarafov S, **Pavlova Z**, Todorov T, Chamova T, Gospodinova M, Tournev I, Mitev V, Todorova A. Founder effect of the Glu89Gln TTR mutation in the Bulgarian population. Amyloid. 2019 Dec 26(4):181-185. doi: 10.1080/13506129.2019.1634539.
   Impact factor (2019): 4.323 **Citations: 10**

2. Yordanova I, **Pavlova Z**, Kirov A, Todorov T, Alexiev A, Sarafov S, Mateva L, Chamova T, Gospodinova M, Mitev V, Tournev I, Todorova A. Monoallelic expression of the TTR gene as a contributor to the age at onset and penetrance of TTR-related amyloidosis. Gene. 2019 Jul 15;705:16-21. doi: 10.1016/j.gene.2019.04.030.
   Impact factor (2019): 2.984 **Citations: 3**

3. **Pavlova Z**, Sarafov S, Todorov T, Kirov A, Chamova T, Gospodinova M, Tournev I, Mitev V, Todorova A. Characterization of population genetic structure of hereditary transthyretin amyloidosis in Bulgaria. Amyloid. 2021 Dec 28(4):219-225. doi: 10.1080/13506129.2021.1935230.
   Impact factor (2021): 6.571 **Citations: 1**

TOTAL IMPACT FACTOR: 13.878

6.2. PARTICIPATIONS IN SCIENTIFIC FORUMS

1. **Pavlova Z**, Kirov A, Sarafov S, Todorov T, Chamova T, Gospodinova M, Mitev V, Tournev I, Todorova A. Prevalence of the Glu89Gln *TTR* mutation in the Bulgarian ATTR patients and investigation of possible founder effect. Poster presentation at ARiA VIII symposium 2019.

2. **Pavlova Z**, Todorov T, Chamova T, Gospodinova M, Mitev V, Tournev I, Todorova A. Population genetic profiling of hereditary transthyretin amyloidosis in Bulgaria and possible non-coding genetic modifiers. Poster presentation at ESHG Conference, June 11-14, 2022, Vienna, Austria (P20.013.C).

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