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Discovery of the connection among age-related macular degeneration, *MTHFR C677T* and *PAI 1 4G/5G* gene polymorphisms, and body mass index by means of Bayesian inference methods

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Abstract: Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the elderly. The aim of this study was therefore to explore the relationship between the presence of multiple gene polymorphisms and 2 distinct advanced 'dry and wet' AMD phenotypes, and to assess gene interactions with the influence of personal factors in a Turkish population as a pilot study.

For the analysis, the data were collected from 73 unrelated participants, grouped as 29 wet and 26 dry AMD patients, and 18 healthy controls. They were all genotyped for the multiple gene polymorphisms in 12 different genes. The data set collected was then analyzed using the Bayesian inference methods and visualized by means of the Bayesian networks.

The results suggest that: 1) the *PAI-1 4G/5G* and *FV G1691A* genes have joint roles in the separation of the 3 groups; 2) both wet and dry AMD can be separated from the control group using the genes *PAI-1 4G/5G*, *FV G1691A*, *FXII V34L*, and *PT G20210A*; 3) although the wet AMD and control groups can be separated by the combination of the *ACE I/D* and *B-fibrinogen-455 G-A* gene polymorphisms, there seems to be no significant effect of the genes on the separation between the dry AMD and control groups; 4) the wet AMD and control groups can be distinguished by the combination of body mass index and the *MTHFR-C677T* and *PAI-1* genes; and 5) there is a correlation between wet AMD and a high body mass index (>30 kg/m^2). It was also found that the impact of body mass index on the disease development seems only in question with the connective availability of the genes *MTHFR C677T* and *PAI-1.* It can be concluded that the combination of the *MTHFR C677T* and *PAI-1 4G/5G* gene polymorphisms in the presence of obesity may increase the risk of wet AMD. In addition, the results further support a complex interplay among genetic and environmental factors in the development of different phenotypes.

Key words: Age-related macular degeneration, body mass index, *MTHFR C677T*, *PAI-1 4G/5G*, gene–gene interaction, Bayesian network

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1. Introduction

Age-related macular degeneration (AMD) is a leading cause of legal blindness among older individuals, most often from industrialized countries [1]. Phenotypically, AMD is classified as 'early' and 'late', which is based on clinical features [2,3]. Late AMD is considered as a disease with 2 different end-stage lesions, i.e. wet and dry. Deposition of debris (termed 'drusen') along Bruch's membrane in the macula is the first evidence of early AMD. An increasing number of large drusen tends to develop in 2 different ways. In the late 'dry' AMD, a geographic loss of retinal pigment epithelium (RPE) occurs in the macular region, while in the late neovascular or 'wet' form of the disease, there is abnormal growth of the choroidal vessels under the retina, which leak fluid and may progress to form a disciform scar [4–6]. Dry AMD has a slower deterioration and better preservation of visual acuity than wet AMD. The neovascular AMD is rapid and the loss of vision is often very severe. The genetic structure and individual risk factors that lead to these diverse phenotypes are currently under investigation. However, it is widely believed that the pathogenesis responsible for AMD is linked to external factors and varying susceptibilities to these external factors may be based upon different genetic backgrounds [7]. The major genetic risk factors for AMD, including the Complement Factor H and HTRA1 variants, appear to predispose to both dry and wet AMD [8,9]. Along with genetic variants, external factors such as age, smoking, sex, and body mass index (BMI) play major roles in the disease pathogenesis [10,11].

It is still not understood why an individual at risk will manifest dry or wet AMD and which factors or interactions can trigger the progression of the maculopathy. The etiology of AMD is complex and the prevalence of both AMD and cardiovascular disease (CVD) is strongly age-dependent. The aim of the present study was therefore to explore the relationship between the presence of multiple gene polymorphisms related to cardiovascular risk factors and the 2 distinct advanced 'dry and wet' AMD phenotypes, and also to assess the gene interactions with personal factors.

2. Materials and methods

2.1. Study population

This study was planned as a prospective case-control study. Informed consent was obtained from each of the participants, and the procedures used conformed to the principles of the Declaration of Helsinki. Patients with AMD (dry and wet) and age-matched controls were recruited from the outpatient clinic at the Department of Ophthalmology, İzmir Education and Research Hospital. None of the AMD or control subjects were related to each other. Both the AMD patients and the control subjects underwent full ophthalmological examinations, including slit-lamp biomicroscopy, fundoscopy, and contact lens biomicroscopic examination of the macula. All eyes with AMD exhibited multiple drusen, geographic atrophy, choroidal neovascular membranes, and vascularized pigment epithelial detachments, but the control subjects did not have any of these signs. Those patients with multiple drusen, geographic atrophy, or both were classified as having dry AMD, but patients with a previous history or any other clinical signs of other macular diseases were not included in the present study. Patients with choroidal neovascular membranes, vascularized pigment epithelial detachments, or both were classified as having wet AMD [12]. Fluorescein and indocyanine green angiography, using a scanning laser ophthalmoscope, was applied to all AMD patients. Choroidal neovascularization was defined as irregular or well-delineated focal or plaque-like zones of indocyanine green hyperfluorescence that persisted or increased in the late phases of the videoangiogram [13]. Cataract patients with no signs of AMD were used as controls. The control group patients had healthy macula and these patients had no diabetes mellitus or hypertension (HT). In the examinations, detailed medical and family histories were obtained from the patients. The patients

and controls filled in a questionnaire about their personal histories (age, sex, essential HT, CVD, diabetes mellitus, smoking habits, overweight, and family medical history). The presence of traditional risk factors in both groups was determined with the criteria standardized and endorsed by the European Society of Cardiology, and a hypertensive condition was attributed when the systolic blood pressure values were $\geq$139 mmHg and/or diastolic blood pressure values were $\geq$89 mmHg in at least 2 separate measurements or when being medicated against HT. The subjects were considered as smokers when consuming more than 5 cigarettes per day or nonsmokers when they had never smoked or had stopped smoking at least 1 year before the sample collection. The BMI was calculated as the weight in kilograms divided by the height in squared meters.

Seventy-three unrelated participants, grouped as 29 wet AMD patients (58–87 years old; mean: 73 $\pm$ 8.0 years), 26 dry AMD patients (49–84 years old; mean: 71 $\pm$ 10.3 years), and 18 healthy controls (54–89 years old; mean: 69 $\pm$ 9.0 years), were included in the present study. The other demographic characteristics of the groups are given in Table 1.

Table 1. The demographic characteristics of the groups (n: number of participants).

Demographic characteristics		Wet AMD (n)	Dry AMD (n)	Control (n)	P-value*
Sex	Men	10	9	6	NS
	Women	19	17	12	NS
Smoking	Yes	23	23	12	NS
	No	6	3	6	NS
BMI (kg/m^2)	18.6–4.9	3	12	9	NS
	25–29.9	8	10	6	NS
	30–39.9	16	4	3	NS
	$>$ 40	2	-	-	-
History of HT	Negative	13	11	7	NS
	Positive	16	15	11	NS
History of CVD	Negative	19	16	14	NS
	Positive	10	10	4	NS

* The nominal variables were compared between the AMD patients and the controls using the chi-square analysis, whereas Fisher's test was used for rare variables where the chi-square test was unsuitable.
NS = not significant.

2.2. Data generation by genotyping

Approximately 10–20 μg of DNA was isolated from peripheral blood samples using the spin column technique (genomic DNA extraction kit; RBC Bioscience, Taipei, Taiwan). The DNA concentration and purity was measured with a UV spectrophotometer (UV-3600; Shimadzu, Kyoto, Japan). The following 12 polymorphisms were evaluated: 2 polymorphisms of the *factor V* gene (*FV G1691A* and *FV A4070G*), 1 polymorphism of the *factor II* gene (*PT G20210A*), 1 polymorphism of the *factor XIII* gene (*FXIII V34L*), 1 polymorphism of the beta-fibrinogen gene (*B-fibrinogen 455 G-A*), 1 polymorphism of the platelet glycoprotein IIIA gene (*HPA L33P*), 1 polymorphism of the *plasminogen activator inhibitor-1* gene (*PAI-1 4G/5G*), 2 polymorphisms of the *methylenetetrahydrofolate reductase* gene (*MTHFR C677T* and *MTHFR A1298C*), 1 polymorphism of the *angiotensin-converting enzyme* gene (ACE I/D), and 2 polymorphisms of genes encoding for apolipoproteins B and E (*Apo B R3500Q* and *Apo E2*, *E3*, and *E4*, respectively). These 12 polymorphisms were analyzed using a commercially available kit (Cardiovascular Disease StripAssay; ViennaLab Labordiagnostika, Vienna, Austria),

according to the manufacturer's instructions. All of the results were confirmed with double replicates. This assay was based on the multiplex polymerase chain reaction and reverse hybridization principle. Table 2 shows the distribution of the genotypes and allele frequencies in wet and dry AMD patients and the control group. Mutant alleles of *ApoB R3500Q* were not found in any case of the patients or controls. Therefore, this mutation was excluded from further analysis. The nominal variables and allele frequencies were compared between the AMD patients and the controls using chi-square analysis. However, Fisher's test was utilized for rare variants where the chi-square test was unsuitable. Statistical analyses were performed using the SPSS 10.0 and $P < 0.05$ was considered to be statistically significant.

2.3. Bayesian inference method

Bayesian networks (BNs) are known as directed acyclic graphs and are effectively used for knowledge representation by exploiting cause-and-effect relationships even in uncertain conditions. BNs are also called 'directed Markov fields' or 'belief networks' [14] and are the probabilistic models in which Bayesian inference methods are used for graphical knowledge representation. In a BN, each node graphically represents an attribute and is called a variable (e.g., age, sex, or expression level of a gene). They graphically encode and represent conditional independence relationships between all or a set of the attributes. In the BNs, connections (arcs) between the nodes (attributes) represent dependency relationships of the variables. It is well known that different configurations of the directions of the arcs may induce the same independencies. In the configurations, the direction of the arcs is not uniquely determined. It can therefore not be expected that the arcs actually reflect the direction of causality, but they show the connections between the related attributes [15].

BNs operate with probabilistic inference methods and hence they reach their decisions from uncertain data (e.g., not true or false). They can also simultaneously handle discrete and continuous variables without any need for arbitrary thresholds. In addition, their semantic presentation also makes them advantageous over other types of classifiers (e.g., artificial neural networks). This type of representation helps display the links between all of the attributes or stratified set(s) of the attributes. Furthermore, it has been shown that they can speed up the learning process by attaching prior knowledge (probabilities) to the attributes and cause less problems in noise and overfitting [14,15]. Therefore, a BN was chosen in this study to investigate and display the relationship between the presence of multiple gene polymorphisms and 2 distinct advanced 'dry and wet' AMD phenotypes, and to assess and visualize the gene interactions with the influence of personal factors. Further information about the BN can be found in [14].

The specification of the attributes used in this study is summarized in Tables 1 and 2. The data set used in the BNs included 11 types of genes, which are called attributes, as listed in Table 2. In addition, 6 more attributes, as listed in Table 1, were included in the analysis in order to assess the effects of personal information.

In this work, the BN software packages PowerCostructor^{TM}and PowerPredictor^{TM}were used for the network construction and classification processes, respectively, where both utilities were based on 'the dependency analysis' [16]. The statistical algorithms examine the network information regarding 2 related variables of the data set and decide whether they are dependent or not. The algorithms also examine how strong the relationships between the 2 variables are. For the construction of the BN, the threshold value (t) was set at minimum, namely 0.1, in order to establish the maximum number of links within the network. In addition, the receiver operating characteristic (ROC) was chosen to determine the optimum models. No discretization method was selected, as there was no continuous variable in the data set under study.

Table 2. Genotype and allele frequencies of the genetic polymorphisms in the participants.

Polymorphism	Wet AMD (n: 29)	Dry AMD (n: 26)	Control group (n: 18)	P-value*
*F*5 (FV Leiden) c.1691G > A[a] (rs6025c)[b]				
GG	25	26	15	
GA	4	-	3	NS
AA	-	-	-	
A allele frequency	0.069	0	0.083	
*F*5 (R2) c.4070A > G[a] (rs1800595)[b]				
AA	23	23	14	
AG	6	3	4	NS
GG	-	-	-	
G allele frequency	0.104	0.058	0.111	
*F*2 (Prothrombin) c.20210G > A[a] (rs1799963)[b]				
GG	28	25	15	
GA	1	1	3	NS
AA	-	-	-	
A allele frequency	0.017	0.019	0.083	
*F*13*A*1 (FXIII) c.143G > T[a] (rs5985)[b]				
VV	19	18	10	
VL	10	5	7	NS
LL	-	3	1	
L allele frequency	0.172	0.212	0.250	
FGB (Fibrinogen) c.455G > A[a] (rs1800790)[b]				
GG	21	14	12	
GA	8	10	5	NS
AA	-	2	1	
*A*allele frequency	0.138	0.269	0.194	
*SERPINE*1 (PAI-I) c.675-676delinsG[a] (rs1799889)[b]				
5G/5G	9	5	2	
5G/4G	11	17	15	NS
4G/4G	9	4	1	
4G allele frequency	0.500	0.481	0.472	
*ITGB*3 (GpIIIa) c.176T > C[a] (rs5918)[b]				
L/L	21	16	9	
L/P	7	7	8	NS
P/P	1	3	1	
P allele frequency	0.155	0.250	0.277	
MTHFR c.677C > T[a] (rs1801133)[b]				
CC	11	17	9	
CT	13	8	8	NS
TT	5	1	1	
T allele frequency	0.397	0.192	0.277	
MTHFR c.1298A > C[a] (rs1801131)[b]				
AA	11	10	10	
AC	17	12	7	NS
CC	1	4	1	
C allele frequency	0.328	0.385	0.250	
ACE g.11417_11704del287[a] (rs4646994)[b]				
I/I	5	7	7	
I/D	13	9	7	NS
D/D	11	10	4	
D allele frequency	0.603	0.558	0.416	
APOE c.388T > C[a] (rs429358)[b] c.526C > T[a] (rs7412)[b]				
2/3	5	4	3	
3/3	20	20	12	NS
3/4	4	2	3	
E3 allele frequency	0.844	0.884	0.833	

* Allele frequencies were compared between the AMD patients and the controls using chi-square analysis. For rare variants for which the chi-square test was unsuitable, we utilized Fisher's test. NS = not significant.
[a] Nomenclature according to the Human Genome Variation Society names in the Single Nucleotide Polymorphism Database (dbSNP) - National Center for Biotechnology Information, [b]: dbSNP ID.

2.4. Dependency analysis in the BN

The dependency analysis examines the information of 2 related nodes from any given data set and decides if the 2 nodes are dependent or not and how close their relationship is. This information is called the conditional mutual information of 2 nodes, A_i and A_j, and can be denoted as:

$$I(A_i, A_j|C) = \sum_{a_i,a_j,c} P(a_i, a_j, c) \log \frac{P(a_i, a_j|c)}{P(a_i|c)P(a_j|c)},$$

where C is a set of nodes and c is a vector (1 instantiation of the variables in C). If $I(A_i, A_j|C)$ is smaller than a certain threshold t, then it can be concluded that A_i and A_j are conditionally independent. The selection of a threshold value for t depends on the data set under study and may be between 0.1 and 50 [16]. The values of $P(a_i, a_j|c)$ may be extracted from the conditional probability tables. For this study, the threshold value (t) was set at minimum, namely 0.1, in order to establish the maximum number of links within the network and consequently investigate the different aspects of the interactions between all of the variables, including the personal information and multiple gene polymorphisms.

3. Results

In order to establish the true nature of the relationship between the attributes and discover a robust set of associative attributes, various scenarios were taken into consideration and analyzed under the following 4 investigations. Each case study presented in the following subsections refers to a modular approach of the Bayesian inference technique. The data inference stages fall into these 4 categories, for each of which a different BN was constructed. This method is called a modular approach because a particular network is built for each case. It should be noted that each network is specific to a particular task and aimed at unveiling different characteristics of the case studies as well as the relationship between the attributes associated with the disease.

The overall results of sensitivity, specificity, and accuracy are presented in Tables 3, 4, and 5. They can be formulated depending on the investigation type. For example, they can be calculated for Investigation 2 as:

Sensitivity = (*number of correctly classified wet_AMD samples / number of all wet_AMD samples*).

Specificity = (*number of correctly classified control group samples / all control group samples*).

Accuracy = (*number of all correctly classified samples / number of all the samples in the data set*).

They can also be formulated similarly for all of the other investigations.

In order to present a generalization ability of the BN models constructed, a 5-fold cross-validation method was adapted, where the data set was divided into 5 subsets, each containing more or less 20% of the samples. Four of these 5 subsets were used first for training a BN-based model, and the BN-based model was then tested over the remaining subset. The procedure was repeated until all of the subsets were tested.

3.1. Investigation 1: General view of the BN configuration with all of the attributes

In this BN, all 3 groups and attributes were included for a network configuration in order to extract the general characteristics of the data set collected. As a result, some remarkable conclusions were observed, such as that a link between smoking and *ACE I/D* polymorphism can be established, as shown in Figure 1. In addition, a connection between the class node group and the genes *PAI-1 4G/5G* and *FV G1691A* was observed. Furthermore, the network configuration appears to indicate that HT is not directly connected to any genetic attribute but only connected to CVD.

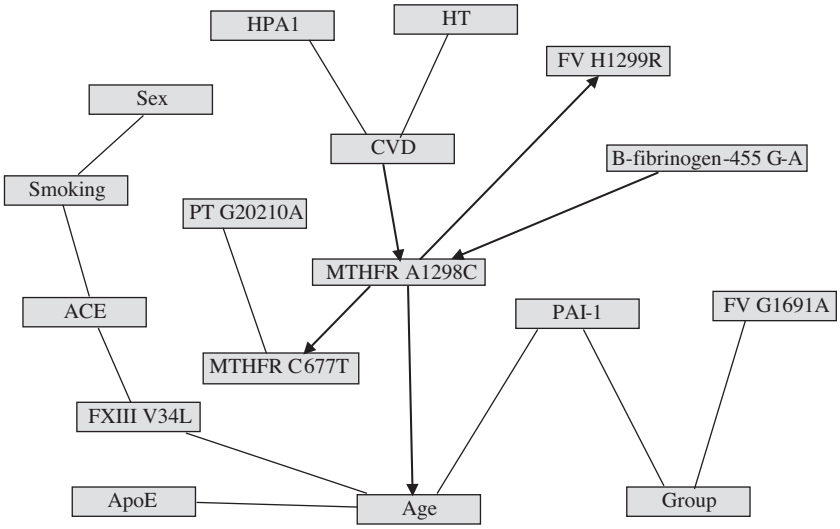

Figure 1. General view of the BN configuration with all of the attributes and groups. It should be noted that in all of the network configurations, the direction of the arcs was determined by the available node ordering and hence does not necessarily show the direction of potential causality.

3.2. Investigation 2: The role of all of the genes in separation between wet AMD patients and control group

This network module was constructed to investigate the effects of the genes on the separation between the wet AMD and control groups, and also to see if there is any genetic difference between the groups with and without the retinopathy disease. The analysis resulted in the BN presented in Figure 2, yielding a predictive accuracy of 83.3%, as shown in Table 3. The ROC area under curve (AUC) measure of the accuracy values and confusion matrix are also shown in Table 4. In Table 4, the AUC indices show the measure of the test's accuracy, which were found to be sensitivity of 89.6% (1 – false control rate) and specificity of 93.5% (1 – false wet_AMD rate). The lift index indicates the ratio between the results predicted using the classification model and the results using no model (predicted randomly).

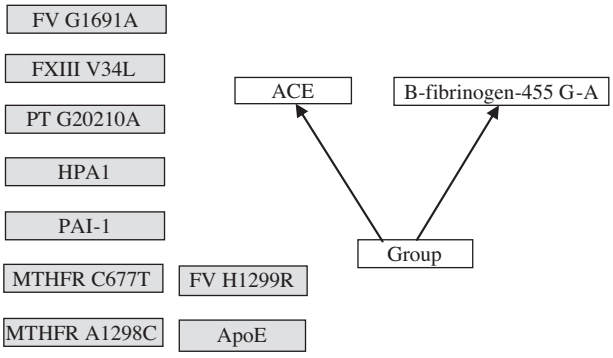

Figure 2. The BN for Investigation 2. After the network construction, the links were established between 2 genes (*ACE I/D* and *B-fibrinogen-455 G/A*) and the class node 'Group' that included the wet AMD and control groups.

Table 3. Predictive accuracy results for all of the investigations.

	BN model for:	Sensitivity (%)	Specificity (%)	Overall accuracy % (±standard error %) at a 95% confidence level
Investigation 2	Investigating the role of all of the genes in separation between the wet AMD patients and the healthy/control groups	81.8	85.7	83.3 (±4.7)
Investigation 3	Investigating the role of all of the genes in separation between the AMD patients (wet and dry AMD) and the healthy/control groups	85.7	25.0	69.0 (±7.0)
Investigation 4	AMD (wet AMD) and the healthy/control groups	83.3	75.0	80.0 (±2.5)

Table 4. For Investigation 2, classification results of the wet AMD patients and the healthy/control group. ROC: receiving operating curve, AUC: area under curve.

For Investigation 2 Classification of the wet AMD patients and control group			
Confusion matrix			
	Wet AMD	Control	Lift index
Wet AMD	9	2	0.681
Control group	1	6	0.793
ROC (AUC) indices			
	Wet AMD	Control	
Wet AMD	0.000	0.935	
Control group	0.896	0.000	

3.3. Investigation 3: The role of all of the genes in separation between the AMD patients (wet and dry AMD) and control group

This investigation was carried out similarly to Investigation 2. However, the wet and dry AMD groups were merged and matched against the control group for this case, in order to see which attributes are associated with AMD in general. The investigation appears to indicate that the AMD patients can be separated easily from the control group by a predictive accuracy of 69% (Table 3). This analysis also generated the BN with the joint roles of the genes *PAI-1*, *FXIII V34L*, *FV G1691A*, and *PT G20210A*, as shown in Figure 3. The ROC (AUC) measure of the accuracy values and confusion matrix are also shown in Table 5. In Table 5, the AUC indices show the measure of the test's accuracy, which were found to be sensitivity of 53% (1 – false control rate) and specificity of 53.7% (1 – false AMD rate). The lift index indicates the ratio between the results predicted using the classification model and results using no model (predicted randomly).

3.4. Investigation 4: The role of high BMI in wet AMD development

The role of BMI in separation between the wet AMD and control groups was investigated to identify a possible association between BMI and wet AMD. The investigation process was pursued in 2 stages. At the first stage,

the attribute '*Group*' was taken as a class node in the BN classifier to prove the casual effect of BMI on the group separation. This classification process resulted in a predictive accuracy of 80% and a BN with the connections between the '*Group*' node and the BMI, *MTHFR C677T*, and *PAI-1 4G/5G* nodes, as depicted in Figure 4. In the second stage, the wet AMD and control groups were taken as the attribute set in the network and the BMI was divided into 2 categories, as normal (BMI_n < 30 kg/m^2) and high (BMI_h ≥ 30 kg/m^2), and this was examined to see whether there is a direct link between the high BMI and the wet AMD group. As seen in Figure 5, the link was established after the process between the 2 nodes. In the network, the exclusion of a direct link with *PAI-1 4G/5G* may be ignored as a specific case; namely, a connection between only the wet AMD group and high BMI was examined. In addition, the link between the wet AMD and control groups may be ignored since it is inevitable, as they already have a limited correlation. It should be noted that the important point is that BMI was included in the network only in Investigation 4. Therefore, the separation processes of Investigations 1, 2, and 3 are not comparable to that of Investigation 4.

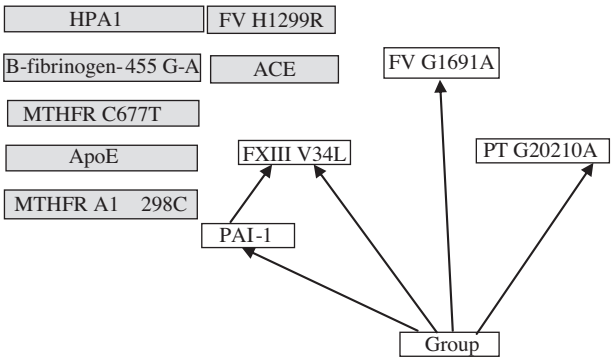

Figure 3. The BN for Investigation 3. The links were established between the class node 'Groups' that included the control group and both the wet and dry AMD groups.

Table 5. For Investigation 3, the classification results of the AMD patients (wet and dry AMD) and the healthy/control group.

For Investigation 3 Classification of the AMD patients (wet and dry AMD) and the healthy/control group			
Confusion matrix			
	AMD	Control	Lift index
Wet AMD	2	1	0.525
Control group	7	0	0.545
ROC (AUC) indices			
	AMD	Control	
AMD patients	0.000	0.537	
Control group	0.530	0.000	

4. Discussion

The most recent advances in gene discovery clearly indicate that AMD has a close link with the interaction of multiple genetic and environmental factors [17]. However, it remains unanswered as to why some patients develop dry AMD while others develop the wet form of the disease. In the last few decades, nonlinear classifiers like BNs have been in very high demand for an accurate and reliable analysis of such medical data sets, which

may contain a considerable amount of uncertainty (e.g., uncertainty in clinical observations or test results). In recent years, a number of research projects have been carried out to exploit the BNs for various types of data analysis and decision making [18–25]. Even though the previous works investigated and developed the Bayesian inference models theoretically, a few of them actually exploited the semantic characteristics of the BNs to derive highly specific information from the links between the attributes. In the proposed work, different types of (modular) BNs for each specific investigation were constructed to investigate and display the relationship between the presence of multiple gene polymorphisms and 2 distinct advanced 'dry and wet' phenotypes of AMD, and to assess and visualize the gene interactions with the influence of personal factors. Each BN that corresponds to a different combination of these cases and attributes can help us to observe effects of various combinations of the attributes on the disease. With this highly specified analysis, where more specific cause-and-effect relationships can be established within the network, the exact role or impact of the genes was studied.

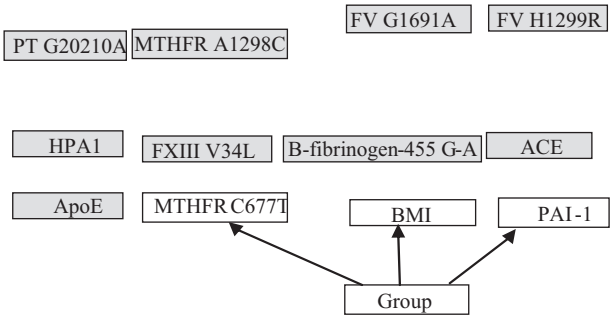

Figure 4. The BN for Investigation 4. The network was constructed with the links between the BMI, 2 genes, and the Group class node (wet AMD and control groups) that yielded a predictive accuracy of 80%.

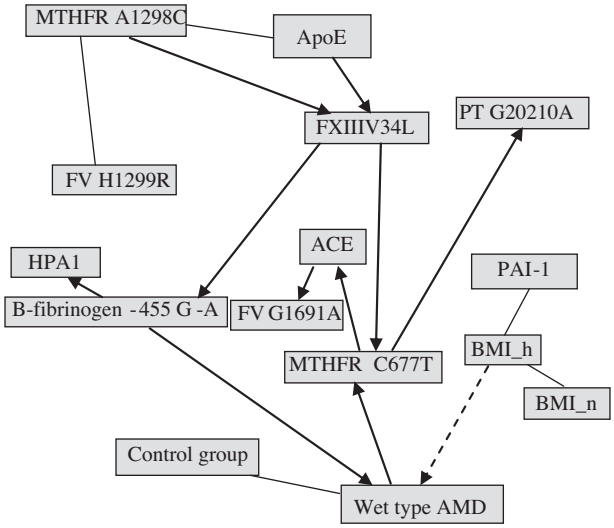

Figure 5. Specific case of the BN for Investigation 4 resulting in a link between the high BMI and wet AMD (dashed line).

It should be noted that the classification process differs from the network construction process, as each one is processed by different utilities of the BN. In the classification process, the priority is given to a model with a more accurate classification, and hence only an optimal group of the attributes were included in the network. As shown in Figure 2, most of the attributes were excluded, not because all of them were unimportant, but because they would not make any further contribution towards the classification accuracy. On the other hand, in the network construction process, the priority is given to discovering more possible connections between the attributes, in which case no class node is selected. The connections between the attributes were analyzed by setting a minimum threshold of t at 0.1 in order to assess all of the possible connections between the attributes.

As far as the clinical tasks of the research are concerned, during normal hemostasis, there is a balance between coagulation and fibrinolysis. Inherited clotting abnormalities such as the *FV G1691A* mutation and *PT G20210A* gene polymorphism are related to unbalanced hemostasis [26,27], and hence the thrombotic tendency. PAI-1 is the major inhibitor of fibrinolysis. The *4G/5G* polymorphism in the *PAI-1* gene promoter region is associated with a higher plasma PAI-1 activity [28]. Elevated plasma concentrations of PAI-1 are associated

with early atherosclerosis [29] and are predictive of mortality after myocardial infarction [30]. In this research, the result of Investigation 1 was the network construction process, which showed that the *PAI-1 4G/5G* and *FV G1691A* genes have joint roles in the separation of the 3 groups (wet AMD, dry AMD, and the control group), as depicted in Figure 1. In addition, in Investigation 3, both AMD cases (wet and dry AMD) can be separated from the control group in association with the joint roles of the genes *PAI-1 4G/5G*, *FV G1691A*, *FXII V34L*, and *PT G20210A*, and the results are depicted in Figure 3 and summarized in Table 3. Although the role of these genes in the pathogenesis of AMD is unknown, the findings of the analyses can be interpreted such that the combination of *PAI-1 4G/5G*, *FV G1691A*, *FXII V34L*, and *PT G20210A* genes may be related to a common risk and represent a characteristic feature shared by both the wet and dry AMD groups. However, it should be noted that this analysis requires further investigation and refinement, as the BN-based model appears biased towards the control group.

In the study, a link between the attribute 'smoking' and the gene *ACE I/D* polymorphism was established. A similar connection was also proven in a previous study [31], as Busquets et al. suggested that *ACE I/D* polymorphisms are associated with the smoking history of an individual. In addition, although the wet AMD and control groups were shown to be able to be separated from each other using the combination of the *ACE I/D* and *B-fibrinogen-455 G-A* gene polymorphisms (Figure 2; Table 3), no significant effect of the genes on the separation between the dry AMD and control groups was observed.

Angiotensin-converting enzyme (ACE) is an enzyme known to catalyze the conversion of angiotensin I to angiotensin II and to deactivate the bradykinin [32]. The *ACE* gene contains a polymorphism based on the presence (insertion [I]) or absence (deletion [D]) within an intron of the 287-bp nonsense DNA domain [33]. The insertion allele of the ACE gene is associated with lower ACE activity in the bloodstream and in the tissues [34]. The *ACE I/D* gene polymorphism was shown to affect angiogenesis [35]. The *ACE I/I* genotype has been found at higher frequencies in the normal than in the dry AMD, and a possible protective role of the polymorphism against the dry form of AMD was suggested [36]. According to a previous study, lower ACE activity enhances the retinal endothelial cell survival in tissue culture [37]. Kostamaa et al. suggested that the insertion allele leads to an enhancement in the endothelial cell survival by ACE inhibition, leading to a predisposition for neovascular AMD [38].

Fibrinogen is a hemostatic factor and acute phase proinflammatory protein. Several studies showed that the A allele of the *-455 G/A* polymorphism in the *fibrinogen* β chain promoter region is associated with an elevated plasma fibrinogen concentration [39,40]. Epidemiological studies have reported a relationship between fibrinogen and AMD [41,42]. It has also been suggested that extravasated fibrinogen might play an important role in the development of the choroidal neovascularization associated with AMD, at least in part, through the function of the vascular endothelial growth factor in an autocrine manner [43].

At this stage, our findings may implicate different mechanisms of the AMD progression. In addition, the results highlight the association of multiple gene polymorphisms with different phenotypes of AMD. A genetic background including the *ACE I/D* and *B-fibrinogen-455 G-A* polymorphisms may mediate the progression to the wet form of early AMD. Considerable vision loss can also result from the features associated with dry AMD, but serious vision loss occurs much more rarely than with wet AMD. The development of effective treatments that reduce the risk of further vision loss and prevent legal blindness makes it more important to predict wet AMD at an early stage so that patients can be treated quickly. Therefore, the prediction of progression of wet AMD is an important effort.

One of the most interesting outcomes of this research is the discovery of the role of BMI in separation between the wet AMD and control groups. In order to reach this conclusion, whether a high BMI may have an effect on developing wet AMD was investigated. At the first stage, the wet AMD group was separated from the control group by the combination of BMI and the *MTHFR-C677T* and *PAI-1* genes (Figure 4). At this stage, it was proven that: 1) the wet AMD group was separated from the control group by a predictive accuracy of 80% and 2) there was a causal link between BMI and at least 1 of the groups, and hence a possible link with AMD. At the second stage, the findings also showed a connection between the wet AMD group and a high BMI (>30 kg/m^2) (Figure 5). It should be noted that the impact of BMI on the disease development is only in question with the connective availability of 2 genes, *MTHFR C677T* and *PAI-1*.

Overweight and obesity are significantly associated with diabetes, high blood pressure, high cholesterol, arthritis, and poor health status [44]. The results from the Blue Mountains Eye Study also suggested an increased risk for the onset of early or dry AMD among individuals outside the normal BMI range [41]. Another study found a relationship between late AMD and pigmentary abnormalities, and a BMI of greater than 30, compared with lean individuals [45], and the Beaver Dam Eye Study also found a significant relationship between BMI and early AMD for women, but not for men [46]. The Age-Related Eye Disease Study also showed that a higher BMI was associated with the prevalence of neovascular AMD [47].

An increased level of PAI-1 has been shown to be associated with obesity and BMI [48]. Moreover, recently increasing evidence demonstrated a proangiogenic role of PAI-1 [49]. In addition, a dose-dependent effect of PAI-1 has been reported in vitro [50] and in vivo in a model of choroidal neoangiogenesis [51].

The C677T polymorphisms in the *MTHFR* gene have been shown to decrease the enzymatic activity, resulting in elevated plasma homocysteine levels [52]. Some studies have shown increased plasma homocysteine in patients with AMD [53]. Another study reported that there were no differences in the plasma homocysteine or folate concentrations in those with wet AMD compared to those with dry AMD [54]. It was also suggested that the *MTHFR C677T* gene polymorphisms are the genetic risk factors for obesity [55].

The results presented in this study suggest that the combination of the *MTHFR C677T* and *PAI-1 4G/5G* gene polymorphisms in the presence of obesity (BMI of greater than or equal to 30 kg/m^2) may increase the risk of wet AMD. A different study reported that increasing physical activity, improving the diet, and sustaining these lifestyle changes can reduce both the body weight and the risk of diabetes [44]. In this context, weight loss may make it possible to delay or prevent the progression of wet AMD in susceptible individuals. The strengths of our study include its prospective analysis of homogeneous groups of different phenotypes of AMD. To the best of our knowledge, this is the first study that demonstrates the combined effect of multiple gene polymorphisms and BMI in the separation of wet AMD from healthy individuals. Having said that, the results support that the manifestation of different phenotypes characteristics in AMD is the result of a complex interplay among genetic and environmental factors. In addition, it is also shown that the progression of AMD, like with many chronic conditions, cannot be explained by a single genetic or environmental risk factor. However, it should be noted that the size of the studied subject population was relatively small and therefore larger-scale prospective studies are needed to confirm these findings.

As a final remark, the study shows how the BNs and their graphical representation of the attributes' connection are suited to such a complex medical problem that involves genetic and individual factors.

In this paper, the combined effects of the genes and the individual risk factors in the 2 main subtypes of AMD (dry and wet) and the controls were investigated using the BNs. Different types of (modular) BNs

for each specific investigation were constructed in order to observe the effects of the different combinations of the attributes on the disease. Through this highly specified analysis, where more specific cause-and-effect relationships were established within the network, the exact role or impact of various sets of the genes was investigated. The results reveal for the first time that the combined effect of multiple gene polymorphisms and BMI was found to be associated with the separation of wet AMD from the healthy individuals. More specifically, it was discovered that the connection among AMD, the *MTHFR C677T* and *PAI-1 4G/5G* gene polymorphisms, and BMI can be made. Understanding the combined effects and interactions between specific genotypes and risk factors may therefore help resolve the complex progression of AMD. If preventive strategies (e.g., weight loss and smoking cessation) become applicable to those who have advanced AMD susceptible gene variants, the public health threats of blindness could be effectively reduced.

5. Conclusions

In this paper, the relationship between the presence of multiple gene polymorphisms and 2 distinct advanced 'dry and wet' AMD phenotypes was studied and the interactions of the genes with the influence of personal factors were also assessed, specifically by taking into account a Turkish population. The computational analysis was carried out using the BN for not only developing the predictive model but also for visualizing the relationships and interactions between the attributes.

The outcome of the study reveals that the *PAI-1 4G/5G* and *FV G1691A* genes were found to be discriminative factors that can be used to distinguish the 3 groups (wet and dry AMD, and the control group). In addition, the genes *PAI-1 4G/5G*, *FV G1691A*, *FXII V34L*, and *PT G20210A* were found to be associated with wet and dry AMD, and they can be used to separate the AMD groups from the control group. When the dry and wet AMD groups were analyzed separately against the control group, although the wet AMD and control groups could be separated by the combination of the *ACE I/D* and *B-fibrinogen-455 G-A* gene polymorphisms, there seemed to be no significant effect of the genes on the separation between the dry AMD and control groups. Moreover, the wet AMD and control groups can be distinguished by the combination of BMI, and the *MTHFR C677T* and *PAI-1* genes. It was also found that there was a correlation between wet AMD and a high BMI (>30 kg/m^2). The impact of BMI on the disease development seems only in question with the connective availability of the genes *MTHFR C677T* and *PAI-1.* This significant outcome highlights the difference between the dry and wet AMD groups, leading to a breakthrough that is expected to help better understand the mechanism of the different forms of AMD, as well as aiding in better treatment and patient management.

This study supports a relationship between life style and AMD, and in particular wet AMD, as a strong correlation was identified between wet AMD and a high BMI (>30 kg/m^2). However, it appears to suggest that the impact of BMI on the disease's development seems only in question with the connective availability of the genes *MTHFR C677T* and *PAI-1.*

This pilot study can conclude that the combination of the *MTHFR C677T* and *PAI-1 4G/5G* gene polymorphisms in the presence of obesity may increase the risk of wet AMD. In addition, the results further support a complex interplay among genetic and environmental factors in the development of different phenotypes.

In order to further generalize the findings or identify genetic variations over the dry and wet AMD groups, a collaborative study is under way to collect patient samples from different regions and carry out a comparative study not only within Turkey, but also across Europe.

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