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ORIGINAL ARTICLE

Does Apolipoprotein E genotype affect cardiovascular risk in subjects with acromegaly?

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Abstract Acromegaly is a syndrome that results when the pituitary gland produces excess growth hormone after epiphyseal closure at puberty. Usually, subjects with acromegaly exhibit a 2- to 3-fold higher mortality rate from diseases that are associated with cardiovascular complications when compared to the normal population. In this study, we therefore aimed to evaluate whether a well-established cardiovascular risk factor, the Apolipoprotein E (Apo E) genotype, contributes to increased risk of cardiovascular complications in subjects with acromegaly. A total of 102 unrelated acromegaly subjects were prospectively included into this case–control association study and constituted our study group. The study group was comparable by age and gender with 200 unrelated healthy subjects constituting our control group. Genomic DNA was isolated from the peripheral blood leukocytes of all subjects and Apo E genotype (codon 112/158) was assessed by melting temperature analyses after using a real-time PCR protocol. The Apolipoprotein E4 allele was found at a significantly higher frequency in the study group when compared with the control group ($P = 0.032$). Subjects with the E2 allele, on the other hand, had significantly increased values in body mass index ($P = 0.004$), waist circumference ($P = 0.001$), C-reactive protein (CRP) ($P < 0.001$), and left-side carotid intima media thickness ($P = 0.025$). The Apolipoprotein E2 genotype might contribute to increased risk of cardiovascular complications in subjects with acromegaly since it is concurrently present with other cardiovascular risk factors such as the left-side carotid intima media thickness and CRP.

Keywords Acromegaly · Cardiovascular complication · Apolipoprotein E · Carotid intima media thickness

Abbreviations

Apo E	Apolipoprotein E
BMI	Body mass index
CIMT	Carotid intima-media thickness
CRP	C-reactive protein
CVD	Cardiovascular disease
EDTA	Ethylenediaminetetraacetic acid
FMD	Flow-mediated dilation
GH	Growth hormone
HDL	High density lipoprotein
IDL	Intermediate density lipoprotein
IGF-1	Insulin-like growth factor-1
LDL	Low density lipoprotein
OGTT	Oral glucose tolerance test
US	Ultrasound
VLDL	Very low density lipoprotein

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Introduction

Acromegaly is a disease that results when the pituitary gland produces excess growth hormone (GH). Cardiovascular manifestations such as arterial hypertension, cardiomyopathy, and valve disease are the most important factors that increase the mortality rate in subjects with acromegaly [1]. This is mainly due to the direct and indirect effects of GH and insulin-like growth factor-1 (IGF-1) on the cardiovascular system that come up as hypercoagulability, abdominal obesity, insulin resistance, alterations in the lipid profile, atherosclerosis, endothelial dysfunction, reduction of pulmonary function, and muscle performance [2].

Apolipoprotein E (Apo E) is a circulating lipoprotein that can be encoded by three different alleles: E2, E3, and E4. Consequently six different genotypes can be found in the general populations which are E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4. E3 is the most commonly found allele of all and encodes the protein variant that contains a cysteine amino acid at position 112 and an arginine at position 158; whereas, the E2 variant contains two cysteine and the E4 variant two arginine at these positions. Apo E has been linked to many important physiological processes and some of them can be altered to a certain degree according to the subjects' genotype. For instance, E2, E3, and E4 have different affinities to receptors such as the very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and other lipoprotein receptors. Defective binding to the low density lipoprotein (LDL) receptor like for the Apo E2 variant, but not for the E3 and E4 variants, can cause lipid metabolism-associated diseases such as type III hyperlipoproteinemia [3, 4]. The Apo E genotype can also affect the efficiency of cholesterol absorption and concomitantly lead to variations of total- and LDL-cholesterol concentration levels in the blood plasma [5].

Elevated Apo E plasma concentrations have been found to contribute to cardiovascular complications in subjects with acromegaly [6]. Besides, circulating Apo E protein was found in the amyloid deposits of pituitary adenomas [7, 8]. However, so far it has never been investigated if the Apo E genotype (codon 112/158) is associated with cardiovascular complications that emerge in acromegaly subjects. In this study, we therefore analyzed the Apo E genotype distribution in a cohort of 102 acromegaly and 200 unrelated control subjects and their probably association with cardiovascular complications.

Materials and methods

Study and control population

The study group was constituted of 102 subjects with acromegaly who were consecutively admitted from January 2007 through December 2009 to the Endocrinology and Metabolic Disease outpatient clinic of Ege University Hospital. Our study protocol was approved by the Ethical Committee of Ege University Medical School and written informed consent was taken from all subjects before participating. The clinical backgrounds of the subjects were reviewed retrospectively. Acromegaly was diagnosed based on medical history, clinical examination, histopathological examination, failure of suppression of serum GH concentrations below 1 ng/ml after 75 g oral glucose load and fasting plasma IGF-1 concentrations above the normal ranges for age and gender. Ages at the time of diagnosis, duration of the disease, and treatment options for acromegaly (surgery, radiotherapy, stereotactic radiosurgery, somatostatin analogs (SSAs), and dopamine receptor agonist) were recorded. 93% of the female and 78% of the male subjects were treated at least with one treatment option, while the remaining (4 female and 11 male) were newly diagnosed naive acromegaly subjects. Number of treated subjects with random GH level of <1 μg/l or nadir GH level of <0.4 μg/l after oral glucose tolerance test (OGTT) was 35; and, 13 of them had controlled disease based on the last consensus criteria to cure acromegaly [9]. Plasma IGF-1 concentrations were under the normal ranges for age and gender in five subjects, and above the normal ranges for age and gender in 17 subjects. Six subjects had hypopituitarism. The study group was comparable by age and gender with 200 unrelated healthy subjects constituting our control group. Control group subjects were recruited from the hospital staff and patients who admitted to the outpatient clinic of Ege University Hospital for a routine health check-up. All of them were found to be free from acute or chronic infections, known ischemic heart disease, peripheral vascular disease, hypertension, dyslipidemia, and any other serious medical problems. Selected study and control group subjects were than further evaluated by physical examination, anthropometric measurements, appropriate laboratory tests, and carotid intima-media thickness (CIMT). Their body weights and heights were measured; body mass index (BMI) was calculated as body weight/height^2 and was expressed in kg/m^2. Blood pressure was recorded as the last of two measurements with the subjects seated using a sphygmomanometer. After an overnight fast the biochemical measurements were performed. Serum concentrations of high-sensitivity C-reactive protein (hs-CRP) were determined by an immunonephelometric assay (N-hs-CRP; Siemens Healthcare Diagnostics, Deerfield, IL); serum total cholesterol, high density lipoprotein cholesterol (HDL-C), LDL-C, triglyceride, glucose, and fibrinogen levels were measured by the Olympus AU 2.700 automated analyzer (Toshiba, Tokyo, Japan). Serum GH concentrations were measured by a chemiluminescent immunometric

assay (Immulite®1000, Siemens Healthcare Diagnostics, Deerfield, IL), and plasma IGF-1 concentrations were measured by an immunoradiometric assay (DSL-2800, Diagnostic Systems Laboratories, Webster, TX). Peripheral venous blood samples for genomic DNA isolation were drawn from an antecubital vein into ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at −20°C until use. For the study and control group subjects measurements of CIMT, which is a sensitive marker to detect early arteriosclerosis, were performed on the mid portion of the common carotid artery by radiologists experienced in ultrasound (US) examinations using the equipment of the Sonoline Elegra Imaging System (Siemens AG, Erlangen, Germany) with a 7.5 MHz linear-array transducer.

Apo E genotyping

Genomic DNA was extracted from peripheral blood leukocytes of the subjects using the MagNA Pure LC DNA Isolation Kit I (Roche Applied Science, Mannheim, Germany). The presences of the Apo E2, E3, and E4 alleles (codon 112/158) were analyzed by the commercial Light Mix ApoE C112R R158C Kit (TIB MOLBIOL, Berlin, Germany). All experiments were carried out on the LightCycler™ 2.0 Instrument (Roche Applied Science, Mannheim, Germany) according to the protocol provided by the kit manufacturer. The Apo E C112R variation was analyzed with a Simple Probe and detected in channel 530; whereas, the R158C variation was analyzed with LightCycler Red 640 and Fluorescein-labeled probes and detected in channel 640. Alleles were identified by the specific melting temperature of the resulting amplicons [10]. Since codon 112 and 158 of Apo E were analyzed simultaneously two Tm values were obtained for each allele (channel 530; 640): for E2, these were 49 and 53°C; for E3, 49 and 63°C; and for E4, 59 and 63°C, respectively.

Statistical analysis

SPSS v.14.0 for Windows (SPSS Inc., Chicago) was used for statistical analysis of the results. A $P < 0.05$ value was accepted as statistically significant. Differences in genotype distribution between the different groups were assessed by logistic regression analysis. Pearson's test was used for calculation of the correlations. Three levels (E2, E3, and E4 carriers) of Apo E allele was formed as mentioned before [11]; wherein E2/E3 was combined with E2/E2 as E2 carrier group, E3/E4 was combined with E4/E4 as E4 carrier group, and E3/E3 served as the reference allele E3 carrier. Biochemical parameters and the different Apo E alleles were compared with the ANOVA test. As, age was positively correlating with the CIMT value as determined before, the same comparison was repeated with co-variation of the age.

Results

The study group consisted of total 102 subjects with acromegaly; i.e., 53 male and 49 female with a mean age of 46.47 ± 10.95 years. Meanwhile, the control group consisted of total 200 unrelated healthy subjects; i.e., 99 male and 101 female with a mean age of 45.48 ± 3.38 years. Clinical characteristics of subjects from the study and control groups are summarized in Table 1.

Apo E haplotype and genotype distribution in the study and control groups are shown in Table 2. The Apo E4

Table 1 Clinical characteristics of the study and control groups

Characteristics	Study group ($n = 102$)	Control group ($n = 200$)	P
Age (years)[a]	46.47 ± 10.95	45.48 ± 3.38	0.115
Gender (male; female)	53; 49	99; 101	
Waist circumference (cm)	99.09 ± 9.58	93.18 ± 5.66	0.067
BMI (kg/m^2)[a]	28.62 ± 5.69	24.2 ± 3.31	0.001
Blood pressure (mmHg)[a]			
Systolic	135.48 ± 24.61	120.18 ± 11.23	0.004
Diastolic	86.77 ± 14.52	72.50 ± 5.65	0.025
Total cholesterol (mg/dl)[a]	192.39 ± 41.34	187.33 ± 13.77	0.129
LDL-C (mg/dl)[a]	115.00 ± 38.23	110.31 ± 11.40	0.507
HDL-C (mg/dl)[a]	51.68 ± 14.15	57.33 ± 4.49	0.058
Triglycerides (mg/dl)[a]	123.45 ± 54.04	115.09 ± 24.07	0.048
Fasting glucose (mg/dl)[a]	105.32 ± 27.46	88.08 ± 3.96	0.015
Fibrinogen (mg/dl)[a]	450.18 ± 101.01	277.12 ± 44.07	0.001
hs-CRP (mg/dl)[a]	0.20 ± 0.23	0.29 ± 0.22	0.004

BMI body mass index, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *IGF-I* insulin-like growth factor-1, *GH* growth hormone

[a] Mean ± standard deviation

Table 2 Distribution of the Apo E haplotypes and genotypes in the study and control groups

Haplotypes/genotypes	Study group[a]	Control group[a]	OR	95% CI	P^b
E2	15 (7.4%)	58 (14.5%)	1.511	0.77–2.94	0.081
E3	172 (84.3%)	321 (80.3%)	R		0.411
E4	17 (8.3%)	21 (5.3%)	3.130	1.331–7.361	**0.032**
E2/E2	1 (1.0%)	0 (0.0%)			
E2/E3	11 (10.8%)	37 (18.5%)			
E2/E4	2 (2.0%)	0 (0.0%)			
E3/E3	75 (73.5%)	142 (71.0%)			
E3/E4	11 (10.8%)	21 (10.5%)			
E4/E4	2 (2.0%)	0 (0.0%)			

Statistically significant *P* values of <0.05 are indicated in bold numbers

R reference, *OR* odds ratio, *CI* confidence interval

[a] % Frequency

[b] Logistic regression analysis

haplotype was found at a significantly higher frequency in the study group (8.3%) when compared to the control group (5.3%) ($P = 0.032$, OR 3.130, 95%CI 1.331–7.361). Whereas, the Apo E2 haplotype was found at a higher frequency in the control group (14.5%) when compared to the study group (7.4%); but this difference was statistically not significant ($P > 0.05$, OR 1.511, 95%CI 0.77–2.94). On the other hand, the Apo E2/E3 and E3/E3 genotypes were found to be higher in the control group; but, a statistical analysis could not performed since the Apo E2/E2, E2/E4, and E4/E4 genotypes were not present in this same group.

Associations of metabolic and hormonal parameters, together with all the other measurements, were also compared to the different Apo E allele carrier situations (E2, E3, and E4) within the study group and are summarized in Table 3. Waist circumference and BMI levels were significantly higher in the Apo E2 carrier group ($P = 0.001$ and 0.004, respectively); but, no significant associations were found between the systolic and diastolic pressure and the Apo E alleles. The same applied to total cholesterol, HDL-C, LDL-C, triglycerides, fasting glucose, GH, IGF-1, and fibrinogen levels which were not associated with the different Apo E alleles (Table 4). However, hs-CRP levels were statistically meaningful lower in Apo E4 and highest in Apo E2 allele carriers in the study group ($P < 0.001$).

A positive correlation was found between the right and left CIMT and the Apo E genotypes of the study group subjects; i.e., Apo E2 carriers had significantly increased CIMT levels when compared to Apo E3 and E4 carriers. As, the correlation was most prominent when considering also the age of the subjects, results were adjusted to age and found to be statistically meaningful; especially, between the left CIMT and the Apo E2 carrier situation ($P = 0.025$).

Table 3 Association between the biochemical and hormonal parameters and the Apo E alleles of the study group

Parameters	Apo E Alleles			P^a
	E2	E3	E4	
Waist circumference (cm)[b]	106.92 ± 11.56	96.40 ± 9.91	91.80 ± 9.47	**0.001**
BMI (kg/m^2)[b]	34.04 ± 6.16	28.57 ± 5.39	28.07 ± 3.64	**0.004**
Systolic pressure	143.00 ± 17.08	133.61 ± 23.93	125.33 ± 27.22	0.164
Diastolic pressure	90.00 ± 12.06	84.00 ± 14.21	78.67 ± 15.05	0.122
Total cholesterol (mg/dl)	171.00 ± 28.15	196.59 ± 39.25	197.33 ± 23.84	0.075
HDL-C (mg/dl)	55.58 ± 7.21	52.55 ± 13.46	49.40 ± 9.76	0.437
LDL-C (mg/dl)[a]	114.45 ± 25.15	114.77 ± 39.66	103.79 ± 32.31	0.602
Triglycerides (mg/dl)	128.92 ± 49.02	127.44 ± 67.41	139.47 ± 83.97	0.824
Fasting Glucose (mg/dl)	110.75 ± 19.59	112.70 ± 35.93	108.67 ± 28.67	0.907
IGF-1 (mg/dl)	526.33 ± 294.12	665.33 ± 395.24	579.67 ± 342.01	0.464
GH (ng/ml)	6.67 ± 11.20	8.66 ± 15.59	3.10 ± 2.24	0.441
Post-glucose GH (ng/ml)	7.24 ± 13.41	7.88 ± 10.75	4.05 ± 4.06	0.658

E2 includes E2/E2 and E2/E3 genotypes; E4 includes E3/E4 and E4/E4 genotypes

BMI body mass index, *HDL* high density lipoprotein, *LDL* low density lipoprotein, *IGF-I* insulin-like growth factor-1, *GH* growth hormone

[a] One-way ANOVA, statistically significant *P* values of <0.05 are indicated in bold numbers

[b] Adjusted to sex and age, all values mean ± standard deviation

Table 4 Association between the Fibrinogen, hs-CRP and CIMT values and the Apo E alleles of the study group

Parameters	Apo E alleles	Mean ± SD	Minimum–maximum	95% CI	*P*
Fibrinogen (mg/dl)[a]	E2	523.285 ± 92.44	389.00–678.00	437.78–608.78	0.069
	E3	448.57 ± 100.63	215.90–760.00	418.34–478.81	
	E4	406.55 ± 93.31	259.00–554.00	334.83–477.00	
hs-CRP (mg/dl)	E2	0.465 ± 0.46	0.03–1.82	0.17–0.75	<0.001
	E3	0.136 ± 0.13	0.01–0.76	0.10–0.16	
	E4	0.033 ± 0.01	0.01–0.06	0.02–0.04	
CIMT right (mm)[a]	E2	1.137 ± 0.28	0.60–1.80	0.95–1.31	0.075
	E3	0.987 ± 0.30	0.50–2.50	0.91–1.06	
	E4	0.856 ± 0.19	0.50–1.20	0.74–0.96	
CIMT left (mm)[a]	E2	1.166 ± 0.25	0.70–1.80	1.00–1.33	0.025
	E3	1.011 ± 0.26	0.50–2.00	0.94–1.07	
	E4	0.877 ± 0.15	0.60–1.10	0.79–0.96	

E2 includes E2/E2 and E2/E3 genotypes; E4 includes E3/E4 and E4/E4 genotypes

CIMT carotid intima-media thickness, *CRP* C-reactive protein, *SD* standard deviation, *CI* confidence interval

[a] CIMT results were repeated with co-variation of the age in univariate analyses

Discussion

It was reported that 60% of all acromegaly subjects mainly die from cardiovascular diseases (CVDs), 25% from respiratory diseases and 15% from malignancies [12]. It is also known that an individual's Apo E genotype can directly affect his/hers plasma Apo E concentrations; which, in turn, is again linked to the elevated incidence of CVDs and high mortality rates [13]. For instance, carriers of the Apo E4 allele have higher plasma concentrations of triglycerides and LDL-C, and exhibit a higher prevalence of CVDs [14]. Previous studies have showed that the Apo E4 allele is also associated with an increased risk of developing hypertension [15], coronary artery sclerosis [16], and myocardial perfusion [17]. In addition, Apo E *knockout* mice exhibited hypertension and endothelial dysfunction [18]. Many studies have demonstrated an association between plasma lipid concentrations and the Apo E genotype; but, in this study we have found that the total cholesterol, HDL-C, LDL-C, and triglyceride levels did not differ between the different Apo E genotypes of acromegaly subjects. Recently, in a large prospective cohort study with 25,630 participants, it was reported that the risk of developing heart diseases was not associated with an individual's Apo E genotype particularly when controlling the LDL-C to HDL-C ratio [11].

Subjects with higher waist circumference and BMI values are candidates for developing metabolic syndromes, and their cardiovascular morbidity and mortality rates increase 1.5- to 3-fold when affected [19]. Our results revealed that carriers of the Apo E2 allele had higher waist circumference and BMI values than those who carried the E3 and E4 alleles. Since, it is known that a relation exists with the Apo E2 allele as an obesity indicator, high BMI can be expected and explained by this fact [20]. The effect of the Apo E2 allele on plasma lipids and coronary disease can also be affected by environmental factors such as dietary intake; e.g., E2 has a potential cardio protective association in Japanese people whose diets are based on low saturated fat [21], whereas it has a proatherogenic association in people living in Western countries whose diets are based on high saturated fat [22].

Fibrinogen and CRP are acute phase reactants and play a significant role as cardiovascular risk factors. Lower levels of CRP were found in subjects with active acromegaly when compared to subjects with well-controlled disease and did not explain increased cardiovascular mortality in acromegaly [23]. Contradictory results have been also reported for the Apo E genotypes and their association with plasma CRP levels [24]. An association between the different Apo E alleles and elevated CRP levels was found; i.e., Apo E genotyping was first performed on statin therapy candidates and later it has been shown that individuals heterozygous or homozygous for the E4 allele had reduced CRP levels [25]. Gronroos et al. have found that the Apo E genotype affects the level of circulating CRP already in children and young adults [26]. Apo E4 has been found to be associated with decreased inflammatory response as measured by CRP [27]. Recently, the relation between atherosclerosis and CRP has been discussed and it has been suggested that an inflammatory environment, such as an acidic pH level, is needed for efficient interaction between CRP and atherogenic LDL during the development of atherosclerosis [28]. We have found an increase of CRP from the Apo E4 to E2 genotype in acromegaly subjects reflected in increased inflammatory response in E2 carriers.

Our finding that carriers of the Apo E4 allele have low CRP levels is actually consistent with most reports for other populations; although, this might not be expected since E4 is associated with increased risk of atherosclerosis [29]. This situation can be probably refer to Apo E being a double-faced protein having different actions in lipid metabolism and inflammation [30]. In addition, Marz et al. suggested that the metabolism of CRP is related to the mevalonate pathway and that in conjunction with this, the mevalonate pathway may be down-regulated in Apo E4 allele carriers resulting in decreased CRP [31].

Increased or decreased GH and IGF-1 levels can affect cardiovascular system function and cause excessive cardiovascular morbidity and mortality. This is because these hormones, which are both expressed in cardiac myocytes, can directly affect myocardium contraction and also lead to cardiomyopathy [32, 33]. It was suggested that GH influences the endothelium via endothelial IGF-1 synthesis or through hypertension, abnormalities of body composition and lipid metabolism [34]. While low levels of IGF-1 increase the risk of ischemic heart disease and stroke, it is argued that high levels of IGF-1 prevent atherosclerosis-associated CVDs [35]. Some commonly accepted atherosclerotic markers are brachial artery flow-mediated dilation (FMD), fibrinogen, procalcitonin, CRP, and CIMT. Measurement of CIMT is a non-invasive method which is widely used as a marker for atherosclerosis since it positively correlates with CVDs. Increased CIMT values in subjects with acromegaly have been shown previously [10]. It has also been reported that CRP >6 mg/l or CIMT >1.25 mm are independently associated with the risk of developing cardiovascular events [36]. A positive correlation between CIMT and acute phase reactants like CRP and procalcitonin has been reported in subjects with acromegaly, indicating procalcitonin as a more sensitive marker than CRP [37]. On the other hand, Paisley et al. reported that acromegalic patients have premature CVD (pressure-related arterial and left ventricular stiffening) rather than atherosclerotic disease [38]. Another study, which did not find a difference in CIMT between acromegaly and control subjects, however, found that CIMT was inversely correlating with serum IGF-1 levels suggesting that low IGF-1 might contribute to atherosclerosis of the carotid arteries in GH-deficient adults [39]. In this study, CIMT, CRP, and fibrinogen levels were analyzed in acromegalic subjects and compared with their different Apo E carrier situations. We found that carriers of the Apo E2 allele, but not E3 and E4, had higher CIMT and CRP values. Although, fibrinogen levels were also high in Apo E2 allele carriers, this result was statistically not significant. Since ours is the first study investigating the Apo E genotype in acromegaly subjects we had to compare our results with a meta-analysis study carried out with healthy subjects, which in contrast found decreased CIMT values in subjects carrying the Apo E2 allele [40]. We cannot explain this inconsistency between healthy and acromegalic subjects carrying the Apo E2 allele. However, as it was reported for ApoE$^{-/-}$ mice that excess GH lead to the development of atherosclerosis [41], our results may also derived from the fact that the effects of the GH differ between the different Apo E genotypes in acromegalic subjects. Nevertheless, we think that more elaborate functional analysis has to be performed to further clarify the mechanisms behind this probable effect.

In conclusion, we evaluated whether the Apo E genotype was associated with acromegaly and its commonly seen clinical characteristic of cardiovascular complications. Interestingly, we found that the prevalence of the Apo E4 allele was significantly higher in subjects with acromegaly when compared to healthy control subjects. Furthermore, acromegaly subjects with the Apo E2 allele had higher BMI, waist circumference, CRP, and CIMT values. These are indicators of increased risk for developing cardiovascular complications; especially, when acromegaly subjects were carriers of the E2 allele. When taken into consideration that acromegaly is usually a lately diagnosed disease and accordingly the begin of its treatment is delayed leading to increased cardiovascular morbidity and mortality rates, it is very important to follow the subjects which are under the risk of developing CVDs. Therefore, a revealed genetic risk factor could provide some predictive information whether an individual is likely to develop cardiovascular complications. Nevertheless, this study's weakness is its limited sample size; but, unfortunately the prevalence of acromegaly is low with an estimated rate of 40–70 cases per million inhabitants and an annual incidence of three to four new cases per million inhabitants [1]. However, all subjects have been followed and their clinical characteristics defined very well in this study.

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