FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

MESTRADO INTEGRADO EM MEDICINA

2013/2014

Tiago Augusto Paiva de Magalhães

Genotype – Phenotype relation in portuguese patients with NOTCH3 mutation

março, 2014

FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

Tiago Augusto Paiva de Magalhães

Genotype – Phenotype relation in portuguese patients with NOTCH 3 mutation

Mestrado Integrado em Medicina

Área: Genética

Trabalho efetuado sob a Orientação de:
Professor Doutor João Paulo Oliveira

Trabalho organizado de acordo com as normas da revista:
Cerebrovascular Diseases

março, 2014

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

Projeto de Opção do 6º ano - DECLARAÇÃO DE INTEGRIDADE

Eu, Tiago Augusto Paiva de Magalhães, abaixo assinado, nº mecanográfico 08080012495, estudante do 6º ano do Ciclo de Estudos Integrado em Medicina, na Faculdade de Medicina da Universidade do Porto, declaro ter atuado com absoluta integridade na elaboração deste projeto de opção.

Neste sentido, confirmo que **NÃO** incorri em plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria de um determinado trabalho intelectual, ou partes dele). Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores, foram referenciadas, ou redigidas com novas palavras, tendo colocado, neste caso, a citação da fonte bibliográfica.

Faculdade de Medicina da Universidade do Porto, 27/3/2014

Assinatura conforme cartão de identificação:

Tiago Augusto Paiva de Magalhães

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO
UNIDADE CURRICULAR PROJETO DE OPÇÃO DISSERTAÇÃO / MONOGRAFIA

Projecto de Opção do 6º ano – DECLARAÇÃO DE REPRODUÇÃO

NOME

Tiago Augusto Faria de Magalhães

CARTÃO DE CIDADÃO OU PASSAPORTE (se estrangeiro)	E-MAIL	TELEFONE OU TELEMÓVEL

NÚMERO DE ESTUDANTE	DATA DE CONCLUSÃO
080801245	2014

DESIGNAÇÃO DA ÁREA DO PROJECTO

Genética

TÍTULO DISSERTAÇÃO/MONOGRAFIA (riscar o que não interessa)

Genotype - Phenotype relation in portuguese patients with NOTCH3 mutation

ORIENTADOR

Prof. Doutor José Paulo Oliveira

COORIENTADOR (se aplicável)

É autorizada a reprodução integral desta Dissertação/Monografia (riscar o que não interessa) para efeitos de investigação e de divulgação pedagógica, em programas e projectos coordenados pela FMUP.

Faculdade de Medicina da Universidade do Porto, 27/3/2014

Assinatura conforme cartão de identificação: Tiago Augusto Faria de Magalhães

In memoriam EP e JM

Genotype-Phenotype relation in portuguese patients with *NOTCH3* mutation

Magalhães, T.[1]

1. Department of Genetics, Faculty of Medicine of the University of Porto

Keywords: CADASIL; genotype-phenotype; genetics; MRI; NOTCH3;

Alameda Prof. Hernani Monteiro 4200-319 Porto, Portugal
Tel. +351 914808488, email: tiagoaugmagalhaes@hotmail.com

ABSTRACT

Background: Cerebral Autossomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is caused by mutations on the NOTCH3 gene on chromossome 19 and can be seen as a plemorphic disease with a high variability in clinical manifestations, both between diferent families and between relatives from a single family. The main clinical manifestations are early onset recurrent subcortical ischemic events, cognitive decline, psychiatric disturbance and migraine with aura. Also, it is thought that cardiovascular risk factors (CVRF) migh modulate the disease and affect its severity. Diagnosis is made by genetic analysis, but Magnetic Ressonance imaging (MRI) is considered a crucial diagnostic tool. Today, there are a large number of reports about CADASIL and is known that there are more than 200 mutations associated to CADASIL phenotype. However, the geographical distribution of these mutations proved to be variable. In Portugal, 90% of CADASIL patients have mutations either on exon 4 or 11. The main aim of this study was to study and identify the major clinical manifestations and MRI presentation from a Portuguese sample with known diagnosis of CADASIL while trying to describe a phenotype-genotype relation within our sample.

Methods: From a pool of over 1000 patients from 28 portuguese medical centres, we selected 11 who were followed in S. João Hospital and from whom we were able to collect clinical information by a questionnaire created by the Department of Genetics of the Faculty of Medicine. We then screened those patients for NOTCH3 mutations.

Results: We found 6 different mutations with 73% or our sample had mutation on exon 4 or 11. The most frequent clinical manifestation was Migraine without aura. Other observed manifestations of the disease were Depression, Transient ischemic attacks and stroke. From the patients with MRI (4), the most frequente findings were áreas of hyperintensity in both the periventricular áreas and centrum semiovale. Seven patients had familiar history of symptoms , the most frequente being early onset stroke. CVRF were present in 6 patients and dyslipidemia was the most frequent CVRF.

Conclusions: The present study permitted us to conclude that there are very significant similarities between portuguese and non-portuguese patients diagnosed with CADASIL despite the different clustering regions of the NOTCH3 mutations. We could not create any significant phenotype-genotype relation and no significant relation was found between clinical manifestation and familiar history or clinical manifestation and CVRF

INTRODUCTION

Cerebral Autossomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL, MIM#125310) is also known by the names of hereditary multi-infarct dementia[1], chronic familial vascular encephalopathy[2] or familial subcortical dementia with arteriopathic leukoencephalopathy[3].

It was possibly first reported by Van Bogaert in 1955, who described two sisters with rapidly progressive sub-cortical encephalopathy of Binswanger's type[4] but only in 1993 Tournier–Lasserve et al used the acronym CADASIL for the first time [5]. Later on, in 1996, linkage studies lead to the identification of the mutated gene *NOTCH3* on chromosome 19 as responsible for the disease [6].

CADASIL may be seen as a pleomorphic disease: the dominant manifestations may vary between different families and the clinical picture and functional course also differ in individuals of the same family[7]. The mean age at onset of symptoms is 45 years and the duration of the disease varies between 10 and 40 years [8]. CADASIL is clinically characterized by Transient Ischemic Attacks (TIA) and subcortical ischemic strokes with accumulating sensory, motor and cognitive deficits[6,9]. Despite the most common clinical findings which include early onset recurrent subcortical ischemic events, cognitive decline, psychiatric disturbance and migraine with aura, in certain patients additional manifestations such as epilepsy or acute encephalopathy can be found[9-12]. Adding to the general symptoms, Adib-Samii et al. reported that cardiovascular risk factors may modulate the disease and increase disease severity[13].

There are no broadly accepted criteria to diagnose CADASIL however, magnetic resonance imaging (MRI) is considered of be crucial for such diagnose since patients with this disease had been reported to have imaging abnormalities found in the brain earlier than expected clinically. Also, in young patients who have CADASIL despite being highly characteristic, cerebral abnormalities are limited [8,14].

Certain specific findings can be observed on cerebral MRI being the frontal lobe the most affected. There are also some studies where higher lesion scores in the temporal white matter were found, predominantly, in the temporopolar region as well as in the external capsule and corpus callosum which are also characteristic findings, as well as periventricular hyperintensity. Lacunar infarcts, as well as microbleeds can also be found, particularly in the basal ganglia, deep white matter, thalamus, cerebellum and the mesencephalon [14-18].

Despite the exact prevalence of CADASIL being unknown, a study from West Scotland demonstrated a prevalence of confirmed CADASIL to be 1.98 per 100 000 adults [19]. According to Orphanet, in Europe, the prevalence of CADASIL has been estimated to range between 1 per 50 000 and 1 per 25 000 (http://www.orpha.net/).

There has been a gradual increase in the number of reports about patients with CADASIL[4].

The *NOTCH3*gene has 33 exons and there are more than 200 different mutations associated with the CADASIL phenotype (Human Gene Mutation Database, HGMD; http://www.hgmd.cf.ac.uk, accessed March, 2014) 95% of which being *missense* [20,21]. The described mutations are located in exons 2-24, which encode the 34 Epidermal Growth Factor repeats (EGFR) that constitute the extracellular domain of the transmembrane receptor encoded by *NOTCH3*[22]. These mutations are strongly stereotyped and either create or destroy a cysteine residue in the EGF-like repeats, leading to an odd number of cysteine residues [22].

While strong clustering of cysteine mutations in EGF-like domains 2–5 (encoded by exons 3 and 4) has been described in several western European countries, the exonic distribution of pathogenic *NOTCH3* mutations shows considerable geographic variation. [23]. In Portugal, approximately 2/3 of the *NOTCH3* mutations identified in patients with CADASIL mapped to exons 4, 11 and more than 90% of Portuguese CADASIL patients have one of the mutations described in theses 2 exons[24].

To date, several studies have been published concerning the familiar and sporadic spectrum of clinical presentation of CADASIL and in most cases it showed to be nearly impossible to establish a clear pattern of symptomatic onset of the disease, in both familiar and sporadic cases. Moreover, only a few reports in the literature gave evidence of genotype–phenotype correlations in CADASIL [25,26].

Therefore, the prime objectives of our study consisted on studying and identifying the major clinical manifestations and MRI imaging presentation in patients from a Portuguese sample with known diagnosis of CADASIL and reported mutation involving cysteine residues on *NOTCH3* gene, describing a phenotype-genotype relation within our sample.

Also, we aimed to study the presence of other factors that are thought to be important in the natural history of CADASIL patients such as the presence of cardiovascular risk factors of the presence of familiar history[8,13].

METHODS

The Department of Genetics of the Faculty of Medicine of the University of Porto was pioneer in the molecular diagnosis of CADASIL in Portugal and since 2004 has a database that now comprises more than 1000 patients referred by 28 different Portuguese medical centers. Patients with clinical diagnosis of CADASIL gave informed consent and underwent peripheral blood collection for extraction of a DNA sample to perform genetic analyses of the *NOTCH3* gene, after a formal request made by a neurologist or geneticist.

After consulting this database we enrolled 169 patients in whom a mutation involving a cysteine residue had been previously found in the *NOTCH3* gene, 115 of these patients were index cases. We selected a total of 22 patients followed in S. João Hospital (HSJ).

From these 22 patients we were able to collect adequate and uniform clinical information of a total of 11 patients after analyses of a questionnaire, created by the Department of Genetics of the Faculty of Medicine, addressed to neurologists and filled by them. [Figure 1]

Arterial hypertension was defined as systolic blood pressure >140 mmHg, or diastolic blood pressure >90 mmHg, diabetes mellitus (DM) was defined according to the World Health Organization (WHO) criteria for diagnosis of DM and dyslipidemia were defined as an age adjusted non-fasting cholesterol >6 mmol/l.

Screening for *NOTCH3* mutations was performed by DNA sequencing analyses. Genomic DNA was extracted from peripheral blood leukocytes using standard protocols.In a sequencial approach according to the known mutational hot-spots, exons 2 to 23 of the *NOTCH3* gene, including their respective exon/intron boundaries, were amplified from genomic DNA samples by the polymerase chain reaction (PCR) in a GeneAmp® PCR System thermocycler (GE Applied Biosystems, Foster City, CA, U.S.A.) using the primers described in Table1. A 25µl mix was prepared with: 1,0µl DNA (100ng/µl); 2,5µl of buffer 10x; 1µl of MgCl2 (25mM); 0,5µl of a dNTP mix at 10mMol (100mM dNTP Set, PCR grade, InvitrogenTM, Carlsbad, CA, U.S.A); 0,5µl of forward primer and 0,5µl of reverse primer (both at 25pmol/µl); and 0,2µl of Taq DNA recombinant polymerase (MBI Fermentas, Vilnius, Lituânia; 5 U/µl). The final volume was obtained by adding 18,8µl of distilled water (B Braun®).

The PCR protocol consisted of an initial denaturation step at 94ºC for 5 minutes followed by 35 cycles of denaturation at 94ºC for a minute, annealing at 65ºC (exons 2, 4 and 13/14), 64ºC (exons 3 and 17), 62ºC (exons 5/6, 11/12, 18/19, 20 and 21-23), 60ºC (exons 7/8 and 16), 59ºC (exons 9/10), 57ºC (exon 15) for another minute, extension at 72ºC also for a minute and a final extension at 72ºC for 10 minutes.

To confirm the effectiveness of the amplification reaction, the PCR products were checked by capillary electrophoresis in a QIAxcel® System (Quiagen, Hilden, Germany).

The products obtained with the PCR reaction were then purified with Agencourt AMPure XP (Beckman Coulter, Brea, CA, U.S.A.) according to the manufacturer's instructions.

Finally, the purified PCR products were automatically sequenced in an ABI PRISM® Genetic Analyzer, using the fluorescence-based BigDye® Terminator sequencing standard.

All NOTCH3 sequence variants identified in the original DNA analyses were confirmed in a second PCR amplification of the corresponding exon.

RESULTS

From the total of 11 patients with previously diagnosed CADASIL and identified *NOTCH3* mutation 8 were index cases and 3 were relatives. Every patient had clinical manifestations of the disease at the time of observation with the mean age at onset being 43,3 ± 18,9 (mean ± SD). The mean age at observation was 53,3 ± 14,1 (mean ± SD; range 43,8 – 62,8). Three of the 11 patients were males and 8 were females and the mean age at observation was respectively 51,7 ± 23,4 and 53,9 ± 11,3. Such differences between sexes were not statistically significant and no difference between sexes was found in what concerns to age at onset.

Six of the 11 patiens had mutations in the exon 11, two of them had mutations in the exon 4, two had a mutation located in exon 8 and 1 had a mutation located in exon 20 (table2).

The clinical manifestations studied are also described in table 2 in order to allow the establishment of a relation between them and the mutation found. Migraine without aura was the most frequently observed (5/11) The second most frequent finding was depression (3/11) followed by both TIA's (2/11) and stroke (2/11).

Only 4 patients had done MRI (table 3) and the most frequent findings were areas of hyperintensity both in periventricular areas (3/4) and centrum semiovale (3/4). From the 3 patients with hyperintensiy in periventricular areas, 2 were found to have the p. R558C mutation and the other, the p. C1009Y mutation. The exact same finding was observed in what concerns the 3 patients with hyperintensity in the centrum semiovale.
Familiar history of symptoms was reported in 7 patients (table 4). The most frequent familiar finding was early onset stroke (5/11). The other more frequent findings were dementia (4/11), followed by migraine without aura (2/11) and depression (2/11). No patient had familiar history of TIA's or migraine with aura. The Odds ratio (OR) presented in the bottom of the table 3 were calculated in order to understand if is there a relation between familiar history and patient clinical manifestations. Familiar history does not seem to be related to our patients' symptoms however, none of these results were found to be statistically significant.
The presence of arterial hypertension, diabetes mellitus, dyslipidemia and the use of tobacco were used to study cardiovascular risk factors (CVRF). Six out of the 11 patients had some sort of cardiovascular risk factor. Dyslipidemia was the most frequent CVRF (4/11). Two patients had arterial hypertension, and other 2 had smoking history. None of all 11 patients had diabetes mellitus.
In table 5 it is shown the relation between CVRF and clinical manifestations. Migraine without aura risk was increased in patients with arterial hypertension (odds ratio [OR] = 1,25; 95% CI, 0,04 to 36,22; P<0,05) and in patients with Diabetes Mellitus (odds ratio [OR] = 4,0; 95% CI, 0,27 to 60,32; P<0,05). Depression risk was increased in patients with Diabetes Mellitus as well (odds ratio [OR] = 1,2; 95% CI, 0,07 to 19,63; P<0,05). However, once again this results are non statistically significant due the valor of 95% CI including 1.
No other CVRF were associated with any clinical manifestation of the disease, including dementia, migraine with aura, early onset stroke or TIA.

DISCUSSION

This study focused on the clinical manifestations of a Portuguese sample of CADASIL patients followed in HSJ by evaluating the presence of major clinical manifestations reported on the disease. We identified 6 different mutations in 4 exons with exon 11 being the most frequently affected. Eight out of the 11 patients studied had mutations either on exon 11 or 4 which are reported to be 2 of the most frequent exons affected in patients with CADASIL[23] and in a previous study, were showed to be the most frequent mutation sites in the Portuguese population[24]. In that study, more than 90% of the patients had mutations either on exon 4 or 11. In our study, the percentage was 73% however we believe that using a larger sample, we would obtain similar values to that found by Ferreira et al..We were not able to establish a relation between clinical manifestations and the different *NOTCH3* mutations found. This absence of any association might be related to the limited number of patients studied which resulted in low statistical robustness. However, even though some studies describe phenotype-genotype correlations, Singhal et al. reported that the genotype cannot be used to predict the phenotype in individuals with CADASIL[27] which makes us think that only with a very larger sample would we have obtained satisfactory results.
The mean age at onset of the symptoms was identical to the reported in other papers[8].
Several studies already reported the major clinical manifestations of CADASIL [6,9-12] as early onset recurrent subcortical ischemic events, cognitive decline, psychiatric disturbance and migraine with aura [9,10], and based on those studies we questioned our sample whether they had experienced said symptomsThe most frequent clinical manifestation amongst our sample was migraine without aura. This finding can be expected in CADASIL patients since most cases present with migraine, mostly with aura though. Moreover, Desmond et al. reported that in a significant number of cases, migraine with aura was the presenting symptom [28]. Nonetheless, Dichgans reported that despite the high prevalence of patients with migraine with aura, most individuals also had experienced episodes without aura[9]. This leads us to question whether the presence of migraine might or not be related to the disease. Although migraine can be expected in patients with CADASIL, due to the reduced number of patients studied we cannot be sure whether this symptom is caused by the disease. Depression is not reported as usual at onset of CADASIL, instead, it appears to be more likely to occur later in the course of the disease as observed by Desmond et al [28]. Also, similarly to our study, where depression was the second most frequent symptom, Bianchi et al. reported a high frequency of psychiatric symptoms like depression. The other two most frequent findings were TIA's and early onset stroke which are consistent with the studies of Dichgans et al. in suggesting ischemic deficits as one of the most frequent findings in patients with CADASIL[9]. The similarity between our findings and the previously reported clinical manifestations suggest that there is none particular difference between the Portuguese population and any other given cohort. Thought further studies with wider samples are recommended

in order to extrapolate our findings to the Portuguese CADASIL patients population.
MRI lesions can be highly characteristic of the disease and are thought to appear before any clinical manifestation, unfortunately we were only able to evaluate 4 patients. [15]. The most frequent findings were areas of hyperintensity in the periventricular caps and in the centrum semiovale. Hervé and Chabriat also reported these findings in patients under 40 years, leading us to believe such abnormalities are, in fact, due to the disease[8]. In addition, a recent report by Choi, JC reports initial MRI lesions in the periventricular areas to subsequently extend into basal ganglia, thalamus and brainstem which we found to be the second most frequent locations for MRI abnormalities in our patients [4]. Once again, and with more emphasis in what concerns to the MRI, we believe a larger sample would be beneficial since the imaging in CADASIL can be rather unique and we only studied 4 patients in 11 possible. Nonetheless, we were capable of, even in such a small sample, encounter the typical findings expected in this disease.
As far as we are concerned, no familiar history of symptomatic relatives could be associated to any kind of symptom in our patients. But it seems to be clear that a well-made family history questionnaire is crucial for the clinical diagnosis of CADASIL as are dialing with and autosomal dominant disease and even in a small sample like ours most of the patients (7/11) had any kind familiar history of symptoms. The distinction between first or other degree relatives was not assessed in the questionnaire which could be important to future investigation results.
Concerning the relation between clinical manifestations and CVRF the sample size issue was again important and probably due to it no statistically significant results were found as well. The risk of migraine with aura was higher both in patients with arterial hypertension or patients with diabetes mellitus. Also, the risk of depression appeared to be elevated in patients with diabetes mellitus. Abid-Samii et al. suggested CVRF as being capable of modulating the disease and increase the disease severity. In that study, no relevant role was found concerning the relation between CVRF and migraine or depression however in a study by Singhal, an association between migraine and high serum levels of homocysteine in patients with CADASIL has been described although not being known the mechanism by which the elevated levels of homocysteine could predispose to this symptom[27]. One problem associated with the study of CVRF was the lack of specific criteria to define different levels of arterial hypertension, as well as dyslipidemia. Also, there were no distinction between currently and ex-smokers.

CONCLUSIONS

The present study permitted us to conclude that there are very significant similarities between portuguese and non-portuguese patients diagnosed with CADASIL despite the different clustering regions of the *NOTCH3* mutations. The mean age at onset of the symptoms in Portuguese patients were similar to earlier studies in different populations and that despite the difficulties in creating a statistical significant study of the major symptoms in CADASIL, our findings were highly identical to those of other studies.
The MRI abnormalities observed can be interpreted as confirmation of such similarities between portuguese and non portuguese CADASIL patients.
We could not create any significant phenotype-genotype relation and no significant relation was found between clinical manifestation and familiar history or clinical manifestation and CVRF. Nonetheless, we consider that the sample size may have precluded further detailed analysis and believe that a larger number of patients should be studied in order for the results to be better correlated and explored.

REFERENCES

1 Sourander P, Walinder J: Hereditary multi-infarct dementia. Lancet 1977;1:1015.
2 Stevens DL, Hewlett RH, Brownell B: Chronic familial vascular encephalopathy. Lancet 1977;1:1364-1365.
3 Davous P, Fallet-Bianco C: [familial subcortical dementia with arteriopathic leukoencephalopathy. A clinico-pathological case]. Revue neurologique 1991;147:376-384.
4 Choi JC: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: A genetic cause of cerebral small vessel disease. Journal of clinical neurology 2010;6:1-9.
5 Tournier-Lasserve E, Joutel A, Melki J, Weissenbach J, Lathrop GM, Chabriat H, Mas JL, Cabanis EA, Baudrimont M, Maciazek J, et al.: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy maps to chromosome 19q12. Nature genetics 1993;3:256-259.
6 Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, Alamowitch S, Domenga V, Cecillion M, Marechal E, Maciazek J, Vayssiere C, Cruaud C, Cabanis EA, Ruchoux MM,

Weissenbach J, Bach JF, Bousser MG, Tournier-Lasserve E: Notch3 mutations in cadasil, a hereditary adult-onset condition causing stroke and dementia. Nature 1996;383:707-710.
7 Andre C: Cadasil: Pathogenesis, clinical and radiological findings and treatment. Arq Neuropsiquiatr 2010;68:287-299.
8 Herve D, Chabriat H: Cadasil. Journal of geriatric psychiatry and neurology 2010;23:269-276.
9 Dichgans M, Mayer M, Uttner I, Bruning R, Muller-Hocker J, Rungger G, Ebke M, Klockgether T, Gasser T: The phenotypic spectrum of cadasil: Clinical findings in 102 cases. Annals of neurology 1998;44:731-739.
10 Chabriat H, Vahedi K, Iba-Zizen MT, Joutel A, Nibbio A, Nagy TG, Krebs MO, Julien J, Dubois B, Ducrocq X, et al.: Clinical spectrum of cadasil: A study of 7 families. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Lancet 1995;346:934-939.
11 Schon F, Martin RJ, Prevett M, Clough C, Enevoldson TP, Markus HS: "Cadasil coma": An underdiagnosed acute encephalopathy. Journal of neurology, neurosurgery, and psychiatry 2003;74:249-252.
12 Opherk C, Peters N, Herzog J, Luedtke R, Dichgans M: Long-term prognosis and causes of death in cadasil: A retrospective study in 411 patients. Brain : a journal of neurology 2004;127:2533-2539.
13 Adib-Samii P, Brice G, Martin RJ, Markus HS: Clinical spectrum of cadasil and the effect of cardiovascular risk factors on phenotype: Study in 200 consecutively recruited individuals. Stroke; a journal of cerebral circulation 2010;41:630-634.
14 van den Boom R, Lesnik Oberstein SA, Ferrari MD, Haan J, van Buchem MA: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy: Mr imaging findings at different ages--3rd-6th decades. Radiology 2003;229:683-690.
15 Auer DP, Putz B, Gossl C, Elbel G, Gasser T, Dichgans M: Differential lesion patterns in cadasil and sporadic subcortical arteriosclerotic encephalopathy: Mr imaging study with statistical parametric group comparison. Radiology 2001;218:443-451.
16 O'Sullivan M, Jarosz JM, Martin RJ, Deasy N, Powell JF, Markus HS: Mri hyperintensities of the temporal lobe and external capsule in patients with cadasil. Neurology 2001;56:628-634.
17 Coulthard A, Blank SC, Bushby K, Kalaria RN, Burn DJ: Distribution of cranial mri abnormalities in patients with symptomatic and subclinical cadasil. The British journal of radiology 2000;73:256-265.
18 Lesnik Oberstein SA, van den Boom R, Middelkoop HA, Ferrari MD, Knaap YM, van Houwelingen HC, Breuning MH, van Buchem MA, Haan J: Incipient cadasil. Archives of neurology 2003;60:707-712.
19 Razvi SS, Davidson R, Bone I, Muir KW: The prevalence of cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (cadasil) in the west of scotland. Journal of neurology, neurosurgery, and psychiatry 2005;76:739-741.
20 Federico A, Bianchi S, Dotti MT: The spectrum of mutations for cadasil diagnosis. Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology 2005;26:117-124.
21 Louvi A, Arboleda-Velasquez JF, Artavanis-Tsakonas S: Cadasil: A critical look at a notch disease. Developmental neuroscience 2006;28:5-12.
22 Joutel A, Vahedi K, Corpechot C, Troesch A, Chabriat H, Vayssiere C, Cruaud C, Maciazek J, Weissenbach J, Bousser MG, Bach JF, Tournier-Lasserve E: Strong clustering and stereotyped nature of notch3 mutations in cadasil patients. Lancet 1997;350:1511-1515.
23 Peters N, Opherk C, Bergmann T, Castro M, Herzog J, Dichgans M: Spectrum of mutations in biopsy-proven cadasil: Implications for diagnostic strategies. Archives of neurology 2005;62:1091-1094.
24 Ferreira S CP, Rocha L, Pinto J, Venâncio M, Glória V, Fernandes G, Viana-Batista M, Oliveira JP: Cadasil: Mutational studies in the portuguese population: European Stroke Conference, 2012, Suppl. 2, pp 1-322.
25 Arboleda-Velasquez JF, Lopera F, Lopez E, Frosch MP, Sepulveda-Falla D, Gutierrez JE, Vargas S, Medina M, Martinez De Arrieta C, Lebo RV, Slaugenhaupt SA, Betensky RA, Villegas A, Arcos-Burgos M, Rivera D, Restrepo JC, Kosik KS: C455r notch3 mutation in a colombian cadasil kindred with early onset of stroke. Neurology 2002;59:277-279.
26 Monet-Lepretre M, Bardot B, Lemaire B, Domenga V, Godin O, Dichgans M, Tournier-Lasserve E, Cohen-Tannoudji M, Chabriat H, Joutel A: Distinct phenotypic and functional features of cadasil mutations in the notch3 ligand binding domain. Brain : a journal of neurology 2009;132:1601-1612.

27 Singhal S, Bevan S, Barrick T, Rich P, Markus HS: The influence of genetic and cardiovascular risk factors on the cadasil phenotype. Brain : a journal of neurology 2004;127:2031-2038.
28 Desmond DW, Moroney JT, Lynch T, Chan S, Chin SS, Mohr JP: The natural history of cadasil: A pooled analysis of previously published cases. Stroke; a journal of cerebral circulation 1999;30:1230-1233.

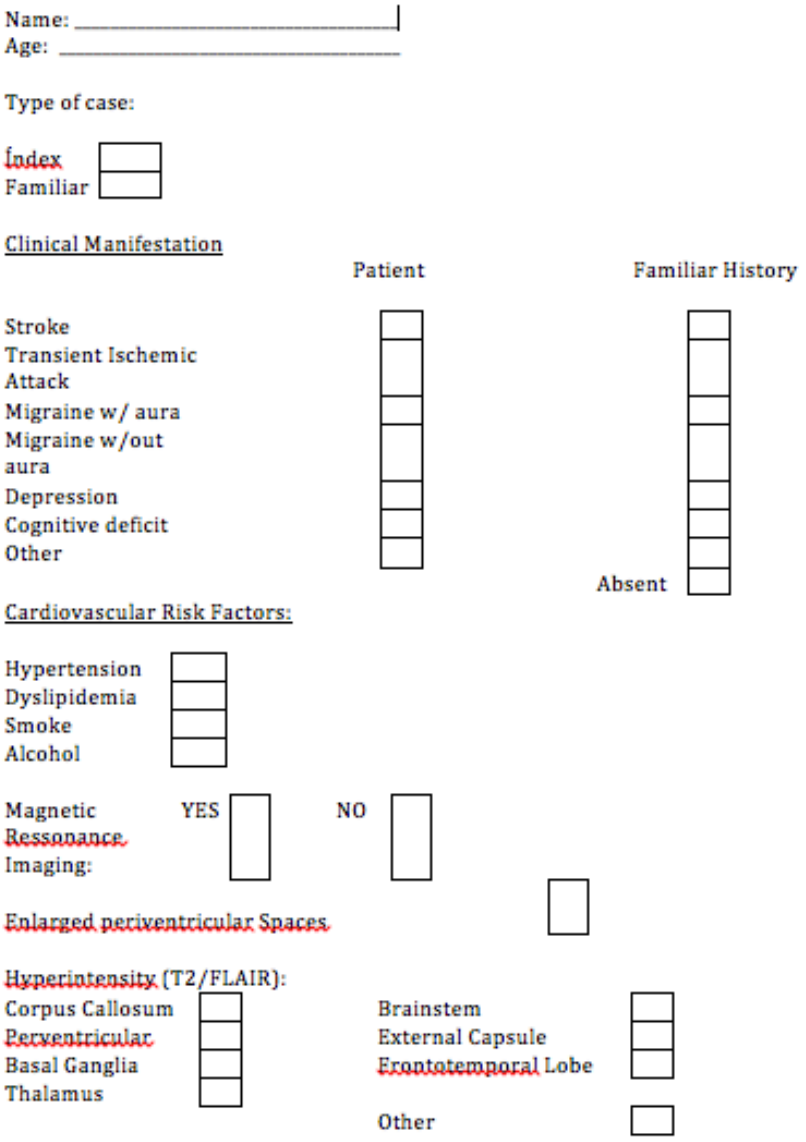

Figure 1: Quetionnaire used created by the Department of Genetics and used to collect cinical data form our patients

Table 1: Primers used in the amplification PCR reaction

Primer code	Primer oligonucleotide sequence	Primer coordinates[a]
CAD 2F	5’-atgcagctgttgcctggtgga-3’	8303- 8323
CAD 2R	5’-ggttgcccaagccacacacat-3’	8619- 8599
CAD 3F	5’-tgtgctgcccaaccaagcca-3’	13430-13449
CAD 3R	5’-actgaccacacccccgacta-3’	13653- 13634
CAD 4F	5’-tagtcgggggtgtggtcagt -3’	13634- 13653
CAD 4R	5’-cctctgactctcctgagtag-3’	14053- 14034
CAD 5F	5’-ctactcaggagagtcagagg-3’	14034- 14053
CAD 6R	5’-tgccctcactaaaaaccatc-3’	14610- 14591
CAD 7F	5’-acaatactcaaggggtgtgg-3’	16479- 16498
CAD 8R	5’-acccacttacaccccattct-3’	17090- 17071
CAD 9F	5’-tgacagtccagcctgtggctga-3’	17466- 17487
CAD 10R	5’-ggctccacacgtagccttatgt-3’	18280- 18259
CAD 11F	5’-tgatgggcagggcctcagat-3’	18539- 18558
CAD 12R	5’-tcgttggacaagagtctgca-3’	19208- 19189
CAD 13F	5’-gcacaagagctgatgcgttatg-3’	20169- 20190
CAD 14R	5’-gctcctggagggaaatgattga-3’	20851- 20830
CAD 15F	5’-ctttcaatcatttccctccag-3’	20827- 20847
CAD 15R	5’-aggctgcatatcagtgtcca-3’	21235- 21216
CAD 16F	5’-cgaatgacagcacggcttat -3’	21406- 21425
CAD 16R	5’-caggcacacagttcaagctt-3’	21757- 21738
CAD 17F	5’-aggcatatcccagtcagact-3’	24110- 24129
CAD 17R	5’-atcagtcatcagagtccgca-3’	24538- 24519
CAD 18F	5’-ccccattcggctcacacta-3’	25385- 25367
CAD 19R	5’-agggacctgattggcttctg-3’	24665- 24684
CAD 20F	5’-ggactcattccaccaaggatgt-3’	25627- 25648
CAD 20R	5’-caagccacacagaaatgtgtgc-3’	25990- 25969
CAD 21F	5’-tactggtagtgcgctgaaca-3’	26360- 26379
CAD 23R	5’-gattacaggtgtgagcta-3’	27327- 27310

Table 2: Clinical Manifestations and NOTCH3 mutations

Patient	Exon	Mutation	Clinical Manifestations						
			Early onset stroke	TIA	Migraine w/ aura	Migraine w/out aura	Depression	Dementia	Other
1	11	R558C	-	x	-	-	-	-	-
2	11	R558C	-	-	-	-	x	-	-
3	11	R558C	-	-	-	x	x	-	-
4	4	R153C	x	-	-	-	-	-	-
5	11	R558C	-	-	-	-	-	-	x
6	4	C168S	-	-	-	x	-	-	-
7	11	R558C	x	-	-	x	-	-	-
8	8	G420C	-	-	-	x	-	-	-
9	8	G420C	-	-	-	x	-	-	-
10	20	C1099Y	-	x	-	-	-	-	-
11	11	R607C	-	-	x	-	x	x	-

Table 3: Imagiological Findings

MRI Findings*	Patient 1 (R558C)	Patient 5 (R558C)	Patient 7 (R558C)	Patient 10 (C1099Y)
Basal Ganglia		x		x
Brainstem		x		
Corpus Callosum		x		
External Capsule	x			
Periventricular	x	x		x
Centrum Semiovale		x	x	x
Thalamus		x		x
Frontotemporal Lobe				
Enlarged periventricular Spaces			x	

* Cerebral structure or location where areas of hyperintensity on T2-weighted, and FLAIR images were observed

Table 4: Relations between clinical manifestations and familar history

Patient	Familiar History						
	Early onset Stroke	TIA	Migraine w/ aura	Migraine w/out aura	Depression	Dementia	Other
1	X	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	x	-	-	x	x	x	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	-	-	-	x	-	x	-
7	x	-	-	-	-	-	-
8	-	-	-	-	-	x	-
9	x	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	x	-	-	-	x	x	x
Odds Ratio	0,80 (0,04 - 17,19)*	0,06 (0,00-3,74)*	0,03 (0,00-2,09)*	0,5 (0,02-14,90)*	0,13 (0,002-5,22)*	0,21 (0,01-8,28)*	0,06 (0,001-2,96)*

*Values shown are OR's with 95% CI's calculated to illustrate the relation between the presence of a familiar symptom and the presence of the same symptom in patients

Table 5: Relations between cardiovascular risk factors and clinical manifestations

	Dementia	Depression	Migraine w/ aura	Migraine w/out aura	Early onset stroke	TIA	Other
Arterial Hypertension	0,13 (0,003-5,22)	0,29 (0,01-6,91)	0,13 (0,003-5,22)	1,25 (0,04-36,22)	0,28 (0,01-9,23)	0,13 (0,003-3,99)	0,13 (0,003-5,22)
Diabetes Mellitus	0,34 (0,01-12,48)	1,2 (0,07-19,63)	0,34 (0,01-12,48)	4,0 (0,27-60,32)	0,8 (0,03-23,18)	0,28 (0,01-9,23)	0,34 (0,01-12,48)
Dyslipidemia	0,03 (0,00-2,09)	0,09 (0,001-5,88)	0,03 (0,00-2,09)	0,21 (0,003-12,52)	0,06 (0,00-3,74)	0,06 (0,00-3,74)	0,03 (0,00-2,09)
Smoking History	0,13 (0,003-5,22)	0,28 (0,01-6,91)	0,13 (0,002-5,22)	0,80 (0,04 - 17,19)	0,13 (0,003-3,99)	0,28 (0,01 - 9,23)	0,13 (0,003-5,22)
Other	0,03 (0,00-2,09)	0,09 (0,001-5,88)	0,09 (0,001-5,88)	0,21 (0,003-12,52)	0,06 (0,00-3,74)	0,06 (0,00-3,74)	0,03 (0,00-2,09)

Values shown are OR's with 95% CI's.

Agradeço ao meu orientador Prof. Doutor João Paulo Oliveira e à Susana Ferreira pelo apoio e disponibilidade prestados ao longo da realização deste trabalho, sem eles não seria possível.

Agradeço ainda à minha família e amigos pelo apoio ao longo da realização desta tese.

Guidelines for Authors
www.karger.com/ced_guidelines

Submission

Manuscripts written in English should be submitted online:

Should you experience any problems with your submission, please contact:

ced@karger.com

Editorial Office 'Cerebrovascular Diseases'
S. Karger AG
P.O. Box
CH-4009 Basel (Switzerland)
Tel. +41 61 306 1437
Fax +41 61 306 1434

Presentation of manuscripts should conform with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals www.doi.org.

Recommended Reading:
– Alexandrov AV: How to Write a Research Paper.
Cerebrovasc Dis 2004;18:135–138

– Alexandrov AV, Hennerici MG: Writing Good Abstracts.
Cerebrovasc Dis 2007; 23:256–259

– How to Improve the Quality of Reports of Parallel-Group Randomized Trials.
The CONSORT Statement. www.consort-statement.org

Conditions

All manuscripts are subject to editorial review.

Manuscripts are received with the explicit understanding that they are not under simultaneous consideration by any other publication. Submission of an article for publication implies transfer of the copyright from the author to the publisher upon acceptance. Accepted papers become the permanent property of *Cerebrovascular Diseases*; and may not be reproduced by any means, in whole or in part, without the written consent of the publisher.

It is the author's responsibility to obtain permission to reproduce illustrations, tables, etc. from other publications.

Conflicts of Interest

Authors are required to disclose any sponsorship or funding arrangements relating to their research and all authors should disclose any possible conflicts of interest. Conflict of interest statements will be published at the end of the article.

Ethics

Published research must comply with the guidelines for human studies and animal welfare regulations. Authors should state that subjects have given their informed consent and that the study protocol has been approved by the institute's committee on human research. Further, they should also state that animal experiments conform to institutional standards.

Categories of Manuscripts

Original Papers are full-length research papers which will be considered for the journal. Articles cover topics relevant to clinical studies. Basic and experimental work appear only if directly related to clinical issues (max. 3,000 words).

Reviews are comprehensive, state-of-the-art papers of important clinical problems. Reviews may be invited by the Editor or they may be unsolicited views (max. 5,000 words). An Abstract is required and should be divided into Background, Summary and Key Messages.

Editorials are usually invited by the Editor (max. 1,000 words). Please send suggestions to the Editor.

Stroke Notes is a category integrating Case Reports, Short Reports, Stroke Vignette, Stroke Opinions. Manuscripts of max. 500 words, 1 figure or table and max. 10 references are considered for publication provided that they describe a very novel observation or add exceptional pertinent, new information into the mechanisms, diagnosis or treatment of a disease.

Translational Research in Stroke is a new section in the journal publishing reviews on experimental results with direct clinical implications (max. 3,000 words). Articles in this section may be accompanied by an invited comment (max. 1000 words).

Imaging is a new section in the journal covering timely papers on cerebrovascular imaging by experts in the field (max. 3,000 words incl. figures and tables, and max. 40 references). Proposals to the Editor are welcome.

Plagiarism Policy

Whether intentional or not, plagiarism is a serious violation. We define plagiarism as a case in which a paper reproduces another work with at least 25% similarity and without citation.
If evidence of plagiarism is found before/after acceptance or after publication of the paper, the author will be offered a chance for rebuttal. If the arguments are not found to be satisfactory, the manuscript will be retracted and the author sanctioned from publishing papers for a period to be determined by the responsible Editor(s).

Arrangement

Title page: The first page of each paper should indicate the title, the authors' names, the institute where the work was conducted, and a short title for use as running head.
NB: Authors wishing to preserve the phonetic meaning of diacritics (PubMed reduces diacritics to their root characters) must spell their names accordingly when submitting manuscripts (e.g. Müller should be Mueller).

Full address: The exact postal address of the corresponding author complete with postal code must be given at the bottom of the title page. Please also supply phone and fax numbers, as well as e-mail address.

Key words: Please supply 3–10 key words in English that reflect the content of the paper.

Abstract: Each paper needs an abstract of 400 words. This should include the following information:

1. *Background:* the problem that prompted the study and the purpose of the study
2. *Methods:* data sources, subjects, design, measurements, data analysis
3. *Results:* most important findings
4. *Conclusions:* implications, future directions.

Abstracts of Reviews: Should be divided into the following subsections: Background, Summary and Key Messages. The Background should provide a brief clinical context for the review and is followed by the Summary, which should include a concise description of the main topics covered in the text. The Key Messages encapsulate the main conclusions of the review.

Footnotes: Avoid footnotes.

Tables and illustrations: Tables and illustrations (both numbered in Arabic numerals) should be prepared on separate pages. Tables require a heading and figures a legend, also prepared on a separate page. For the reproduction of illustrations, only good drawings and original photographs can be accepted; negatives or photocopies cannot be used. Due to technical reasons, figures with a screen background should not be submitted. When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm) or provide crop marks. Electronically submitted b/w half-tone

and color illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800–1,200 dpi.

Color Illustrations

Online edition: Color illustrations are reproduced free of charge. In the print version, the illustrations are reproduced in black and white. Please avoid referring to the colors in the text and figure legends.

Print edition: Up to 6 color illustrations per page can be integrated within the text at CHF 800.00 per page.

References

In the text identify references by Arabic numerals [in square brackets]. Material submitted for publication but not yet accepted should be noted as [unpublished data]; and not be included in the reference list. The list of references should include only those publications which are cited in the text. Do not alphabetize; number references in the order in which they are first mentioned in the text. The surnames of the authors followed by initials should be given. There should be no punctuation other than a comma to separate the authors. Preferably, please cite all authors. Abbreviate journal names according to the Index Medicus system. Also see International Committee of Medical Journal Editors: Uniform requirements for manuscripts submitted to biomedical journals (www.icmje.org).

Examples

(a) Papers published in periodicals:

Chatel J-M, Bernard H, Orson FM: Isolation and characterization of two complete Ara h 2 isoforms cDNA. Int Arch Allergy Immunol 2003;131:
14–18.

(b) Papers published only with DOI numbers:

Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol DOI: 10.1159/000063858.

(c) Monographs:

Hennerici M, Sitzer G, Weger H-D: Carotid Artery Plaques:
Pathogenesis – Development – Evaluation – Treatment. Basel, Karger, 1988.

(d) Edited books:

DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, Dubois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124–137.

Reference Management Software: Use of EndNote is recommended for easy management and formatting of citations and reference lists.

Digital Object Identifier (DOI)

S. Karger Publishers supports DOIs as unique identifiers for articles. A DOI number will be printed on the title page of each article. DOIs can be useful in the future for identifying and citing articles published online without volume or issue information. More information can be found at www.doi.org

Supplementary Material

Supplementary material is restricted to additional data that are not necessary for the scientific integrity and conclusions of the paper. Please note that all supplementary files will undergo editorial review and should be submitted together with the original manuscript. The Editors reserve the right to limit the scope and length of the supplementary material. Supplementary material must meet production quality standards for Web publication without the need for any modification or editing. In general, supplementary files should not exceed 10 Mb in size. All figures and tables should have titles and legends and all files should be supplied separately and named clearly. Acceptable files and formats are: Word or PDF files, Excel spreadsheets (only if the data cannot be converted properly to a PDF file), and video files (.mov, .avi, .mpeg).

Author's Choice™

Karger's Author's Choice™ service broadens the reach of your article and gives all users worldwide free and full access for reading, downloading and printing at www.Karger.com. The option is available for a one-time fee of CHF 3,000.00, which is a permissible cost in grant allocation. More information can be found at www.karger.com/authors_choice.

NIH-Funded Research

The U.S. National Institutes of Health (NIH) mandates under the NIH Public Access Policy that final, peer-reviewed manuscripts appear in its digital database within 12 months of the official publication date. As a service to authors, Karger submits your manuscript on your behalf to PubMed Central (PMC) immediately upon publication. It usually receives a PMCID within approximately a month and will appear in PMC after 12 months. For those selecting our premium Author's Choice™ service, the usual embargo will be overridden, accelerating the accessibility of your work. More details on NIH's Public Access Policy are available here.

Self-Archiving

Karger permits authors to archive their pre-prints (i.e. pre-refereeing) or post-prints (i.e. final draft post-refereeing) on their personal or institution's servers, provided the following conditions are met: Articles may not be used for commercial purposes, must be linked to the publisher's version, and must acknowledge the publisher's copyright. Authors selecting Karger's Author's Choice™ feature, however, are also permitted to archive the final, published version of their article, which includes copyediting and design improvements as well as citation links.

Page Charges

There are no page charges for papers of 4 or fewer printed pages (including tables, illustrations and references). Each additional complete or partial page is charged to the author at CHF 325.00. The allotted size of a paper is equal to approx. 13 manuscript pages (including tables, illustrations and references).

Proofs

Unless indicated otherwise, proofs are sent to the corresponding author and should be returned with the least possible delay. Alterations other than the correction of printer's errors are charged to the author.

Reprints

Order forms and a price list for reprints are sent with the proofs. Orders submitted after the issue is printed are subject to considerably higher prices.