Exon 4 Mutations Determination in NOTCH3 Gene for Cryptogenic Ischemic Stroke

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Abstract

Background: Ischemic stroke in young adults is often classified as cryptogenic (CIS) despite extensive investigation into potential causes. Genetic factors, including mutations in the NOTCH3 gene, are implicated in cerebrovascular diseases such as CADASIL. This study aimed to assess the clinical presentation, vascular risk factors (VRFs), and genetic contributions to CIS in young Algerian adults. Methods: A cross-sectional study was conducted with 42 CIS patients (aged ≤50 years), diagnosed based on the TOAST classification. Data on lifestyle-related VRFs, such as tobacco use and substance consumption, were obtained via interviews. Genotyping was performed on ten single nucleotide polymorphisms (SNPs) in exon 4 of the NOTCH3 gene, a region commonly associated with CADASIL mutations. Results: The mean age of participants was 38.31 ± 8.51 years, with a significant gender difference in age (P = 0.021). Tobacco consumption was a notable risk factor (P = 0.002). However, no mutations were detected in the targeted SNPs of exon 4 of the NOTCH3 gene, either in heterozygous or homozygous states. Conclusion: This suggests that mutations in exon 4 of the NOTCH3 gene may not be involved in the pathogenesis of CIS in this cohort or that unique genetic variants may exist in the Algerian population. The absence of mutations in the analyzed region underlines the need for further research, including comprehensive genetic analysis targeting the entire coding region of the NOTCH3 gene. Larger studies are essential to validate these findings and improve diagnostic, therapeutic, and preventive strategies for CIS in young adults.

Keywords: Cryptogenic ischemic stroke, NOTCH3 gene, CADASIL, young adults, vascular risk factors

Significance | This study highlights the genetic and vascular risk factors for cryptogenic ischemic stroke in young adults, emphasizing prevention and personalized treatment strategies.

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Introduction

Acute ischemic stroke (AIS) represents a major global health burden due to its high incidence, mortality, and long-term disability rates. It is primarily caused by the occlusion of cerebral arteries, leading to brain ischemia and subsequent neuronal damage (Phipps & Cronin, 2020). Among the subtypes of ischemic stroke, cryptogenic stroke has garnered increasing attention due to its unclear etiology and the challenges it presents in diagnosis and management. Cryptogenic strokes, accounting for approximately 20–30% of all ischemic strokes, are characterized by the absence of identifiable causes such as large artery atherosclerosis, cardioembolism, or small vessel disease (Schulz, 2019). This ambiguity underscores the critical need for advancing research into their underlying mechanisms, risk factors, and potential preventive strategies.

The young adult population experiences a unique burden of ischemic stroke due to its devastating impact on productivity and

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quality of life. While the overall prevalence of ischemic stroke is higher in older adults, young adults are increasingly affected, often presenting with distinct risk factors and etiologies compared to their older counterparts (Stack & Cole, 2018). Notably, cryptogenic ischemic strokes are disproportionately more common in younger individuals, with studies highlighting their association with novel genetic and environmental risk factors (Divišová et al., 2020; Yuan et al., 2022).

Emerging evidence suggests a monogenic basis for a subset of young-onset cryptogenic strokes, particularly those linked to cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Yuan et al., 2022). CADASIL, caused by mutations in the NOTCH3 gene, is a hereditary cerebral small vessel disease characterized by recurrent strokes, cognitive decline, and migraines (Papakonstantinou et al., 2019). Mutations in the NOTCH3 gene disrupt the extracellular domain of the Notch3 receptor, leading to the formation of protein aggregates in vascular smooth muscle cells (Dupré et al., 2024). These aggregates are believed to play a central role in vascular pathology and disease progression (Wang et al., 2024). Furthermore, the role of NOTCH3 mutations extends beyond CADASIL, implicating them in broader phenotypes of small vessel diseases, underscoring the importance of genetic studies in understanding stroke pathogenesis (Mizuta et al., 2024).

Despite significant advancements, the exact mechanisms through which NOTCH3 mutations contribute to ischemic stroke remain incompletely understood. The retention of mutant NOTCH3 aggregates in the endoplasmic reticulum has been shown to impair cellular proliferation, mitochondrial function, and mitophagy, further exacerbating vascular dysfunction (Takahashi et al., 2010; Wang et al., 2024). Additionally, pericytes, critical cells in maintaining cerebrovascular homeostasis, have been identified as key players in the pathogenesis of CADASIL, highlighting the multifactorial nature of this disease (Ruchoux et al., 2021).

Epidemiological studies emphasize the interplay of traditional and nontraditional risk factors in the development of ischemic stroke in young adults. Risk factors such as hypertension, diabetes mellitus, obesity, and smoking are well-documented, while migraine, hypercoagulability, and genetic predisposition are increasingly recognized as critical contributors (George, 2020; Martinez Majander et al., 2021). The unique challenges of cryptogenic stroke lie in its diagnostic complexity and recurrence risk. Advanced imaging techniques, prolonged cardiac monitoring, and genetic screening have emerged as essential tools for identifying underlying causes and preventing recurrent strokes in this population (Yaghi et al., 2017; Vera et al., 2023).

Moreover, the link between cryptogenic stroke and prothrombotic states has been explored, with evidence suggesting an increased risk in individuals with autoimmune disorders, antiphospholipid syndrome, and other hypercoagulable conditions (Karttunen et al., 2002; Leppert et al., 2024). The contribution of lifestyle factors, including diet, physical inactivity, and stress, further complicates the risk profile for cryptogenic stroke, particularly in young adults (Ekker et al., 2023).

Research on CADASIL and its molecular underpinnings has opened new avenues for therapeutic interventions. Pharmacological targeting of the Notch signaling pathway, modulation of protein aggregation, and strategies to restore mitochondrial function represent promising approaches under investigation (Papageorgiou et al., 2024). However, challenges remain in translating these findings into clinical practice due to the rarity of CADASIL and the heterogeneity of cryptogenic stroke etiology.

Cryptogenic strokes and their association with genetic factors like NOTCH3 mutations underscore the need for multidisciplinary approaches to understanding and managing ischemic strokes in young adults. Future research should focus on integrating genetic, molecular, and epidemiological insights to develop personalized prevention and treatment strategies. These efforts are vital to reducing the burden of cryptogenic strokes and improving outcomes for affected individuals.

2. Materials And Methods

2.1 Study Population

Participants were recruited over three years (2020–2023) from Eastern Algeria, specifically from the Neurology Department at the University Hospital Center Benbadis of Constantine and the EPHP Bachir Mentouri healthcare facility. A total of 42 young adult patients (<50 years old) diagnosed with cryptogenic ischemic stroke (CIS) were included. The diagnosis was based on the TOAST classification, excluding atherosclerosis, cardioembolism, or small vessel disease as potential causes (Schulz, 2019; Kernan et al., 2014). A range of assessments—MRI, electroencephalogram (EEG), electrocardiogram (ECG), transesophageal echocardiogram (ETO), and echocardiogram with Doppler—was performed to rule out alternative stroke etiologies (Divišová et al., 2020).

Patient demographics, clinical history (e.g., age, gender, family health records), and vascular risk factors (VRFs) were documented using standardized case report forms (Ekker et al., 2023). Hypertension was defined as systolic blood pressure >140 mmHg or diastolic pressure >90 mmHg, or the use of antihypertensive medication (George, 2020). Hypercholesterolemia was identified by LDL cholesterol levels ≥3.4 mmol/L or non-HDL cholesterol ≥4.1 mmol/L, or through lipid-lowering therapy. Smoking history was recorded in pack-years, and diabetes was defined as a fasting glucose level >7.0

mmol/L or prior diagnosis (Olesen et al., 2019; Leppert et al., 2024).

2.2 Ethics Statement

This study complied with the principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the Dr. Benbadis University Hospital Center in Constantine, Algeria (Reference Number: CE/CHUC/11/12/2024). Written informed consent was collected from all participants, including both patients and controls, prior to their inclusion in the study.

2.3 DNA Extraction

Genomic DNA was isolated from peripheral blood leukocytes using a salting-out method with NaCl. Blood samples (5–7 mL) were collected into EDTA vacutainer tubes via venipuncture. The DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 8000), ensuring A260/A280 ratios between 1.8 and 2.0 for high-quality extractions (Yuan et al., 2022).

2.4 Genetic Analysis

Genetic analysis targeted specific single nucleotide polymorphisms (SNPs) in exon 4 of the NOTCH3 gene, with a fragment size of 392 bp. SNPs screened included rs1043994, rs1555729468, rs2145441541, rs2145441610, rs1568361851, rs28933697, rs1555729486, rs1599394806, rs28933696, and rs2145441996 (Papageorgiou et al., 2024).

PCR amplification was carried out using the Veriti™ 96-Well Fast Thermal Cycler. The primers used for amplification were forward (5'-CTCACTCACCAGGAAGACAG-3') and reverse (5'-GGGTGTGGTCAGTCCTAAACT-3'). The cycling conditions included an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 56°C for 40 seconds, and extension at 72°C for 30 seconds. The process concluded with a final extension step at 72°C for 7 minutes. Amplification success was verified through electrophoresis on a 2% agarose gel stained with GelRed (Thermo Fisher). The PCR products were then purified using the EXOSAP enzyme (a mixture of 5 µL PCR product and 2 µL enzyme), incubated at 37°C for 15 minutes to allow digestion, and subsequently inactivated at 80°C for 15 minutes. Sequencing of the purified products was performed using the ABI3500xL Genetic Analyzer in conjunction with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) (Mizuta et al., 2024; Wang et al., 2024).

2.5 Bioinformatics Analysis

The obtained nucleotide sequences were analyzed using BioEdit (version 7.2.5) and MEGA (version 11.0.13) software (Hall, 1999). SNP variants were cross-referenced with available databases to ensure accuracy and interpret potential pathogenicity.

2.6 Statistical Analysis

Data were analyzed using SPSS software (version 22.0; SPSS, Chicago, Illinois). The Shapiro-Wilk test assessed the normality of data distribution. Continuous variables with non-normal distributions were reported as medians with interquartile ranges, while normally distributed variables were presented as means ± SD. Group comparisons employed the Mann-Whitney U test for nonparametric data, and the chi-square or Fisher's exact test for categorical data. A p-value <0.05 was considered statistically significant (Karttunen et al., 2002; Martinez Majander et al., 2021).

3. Results

A total of 42 patients (13 males and 29 females) with defined CIS, confirmed by MRI, and aged ≤50 years were included in this study. All participants were admitted to the Department of Neurology at the University Hospital Center Benbadis of Constantine between 2020 and 2023. Demographic details, baseline characteristics, and risk factors are summarized in Table 1. The mean age of the cohort was 38.31 ± 8.51 years, with males having a higher average age (42.77 ± 8.11 years) compared to females (36.31 ± 8.03 years), a statistically significant difference (P = 0.021). Stroke severity assessed by the NIHSS score was higher in women (5.48 ± 5.16) compared to men (4.08 ± 6.34), although the difference was not statistically significant (P = 0.453). The most common traditional vascular risk factors were arterial hypertension (21.43%), smoking (14.29%), and obesity (11.90%), while hyperlipidemia and diabetes mellitus were less frequent (both 4.76%). No alcohol consumption or drug use was reported. Except for tobacco use, which was significantly more prevalent among men (P = 0.002), no other significant sex-based differences were observed. Migraine was reported in 47.62% of the cohort, and snoring in 19.05%, while none of the patients had dementia.

Genotyping of the NOTCH3 gene using direct Sanger sequencing revealed no mutations in any of the ten selected polymorphisms in exon 4. No heterozygous or homozygous mutations were detected in the cohort, and sequencing electropherograms confirmed the wild-type SNPs (Table 2, Figure 1).

4. Discussion

This study provides valuable insights into the clinical and biological profile of early-onset CIS in Algeria, emphasizing the role of traditional vascular risk factors and potential genetic contributions. The findings underscore the importance of comprehensive risk factor management and the integration of genetic and radiological data for accurate diagnosis and personalized treatment. Further research is needed to elucidate the molecular mechanisms underlying CIS and identify novel therapeutic targets to improve outcomes in this population.

This study represents the first prospective characterization of the clinical and biological profile of early-onset cryptogenic ischemic stroke (CIS) in Algeria. It also explores potential vascular and genetic risk factors that could influence the clinical phenotype of this pathology. Early-onset CIS is considered the most prevalent

Table 1. Demographic, baseline characteristics, and risk factors **N**- Number of subjects

	All	**Males**	**Females**	***P* value**
N	42	13 (30.95%)	29 (69,05%)	N/A
Age (years, mean ± SD)	38.31 ±8.51	42.77 ±8.11	36.31 ±8.03	**0.021**
Admission NIHSS (mean ± SD)	5.05±5.51	4.08 ±6.34	5.48 ±5.16	0.453
Smoking (%)	6 (14.29%)	6 (46.15%)	-	**0.002**
Alcohol drinking (%)	-	-	-	/
Drug consummation (%)	-	-	-	/
Arterial hypertension (%)	9 (21.43%)	1 (7.69%)	8 (27.59%)	0.218
Hyperlipidemia (%)	2 (4.76%)	-	2 (6.90%)	0.491
Obesity (BMI >30)	5 (11.90%)	1 (7.69%)	4 (13.79%)	0.528
Diabetes mellitus (%)	2 (4.76%)	1 (7.69%)	1 (3.45%)	0.540
Migraine	20 (47.62%)	3 (23.08%)	17 (58.62%)	0.151
Dementia	-	-	-	/
Snoring	8 (19.05%)	1 (7.69%)	7 (24.14%)	0.274
Family history of stroke	5 (11.90%)	-	5 (17.24%)	0.181

Table 2. Results of *NOTCH3* SNPs genotyping.

SNP	**Description**	**Pathogenicity status**	**wild-type homozygous heterozygous mutant homozygote**	**(n,%)**
***rs*1043994**	c.606A>G (p.Ala202=)	Benign	AA	42 (100%)
			AG	-
			GG	-
***rs*1555729468**	c.602G>A(p.Cys201Tyr)	Likely pathogenic	GG	42 (100%)
			GA	-
			AA	-
***rs*2145441541**	c.581G>T(p.Cys194Phe)	Pathogenic	GG	42 (100%)
			GT	-
			TT	-
***rs*2145441610**	c.566A>G(p.Tyr189Cys)	Pathogenic	AA	42 (100%)
			AG	-
			GG	-
***rs*1568361851**	c.548G>T(p.Cys183Phe)	Likely pathogenic	GG	42 (100%)
			GT	-
			TT	-
***rs*28933697**	c.544C>T(p.Arg182Cys)	Pathogenic	CC	42 (100%)
			CT	-
			TT	-
***rs*1555729486**	c.521G>T(p.Cys174Phe)	Pathogenic	GG	42 (100%)
			GT	-
			TT	-
***rs*1599394806**	c.520T>C(pCys174Arg)	Likely pathogenic	TT	42 (100%)
			TC	-
			CC	-
***rs*28933696**	c.505C>T(p.Arg169Cys)	Pathogenic	CC	42 (100%)
			CT	-
			TT	-
***rs*2145441996**	c.485G>T(p.Cys162Phe)	Likely pathogenic	GG	42 (100%)
			GT	-
			TT	-

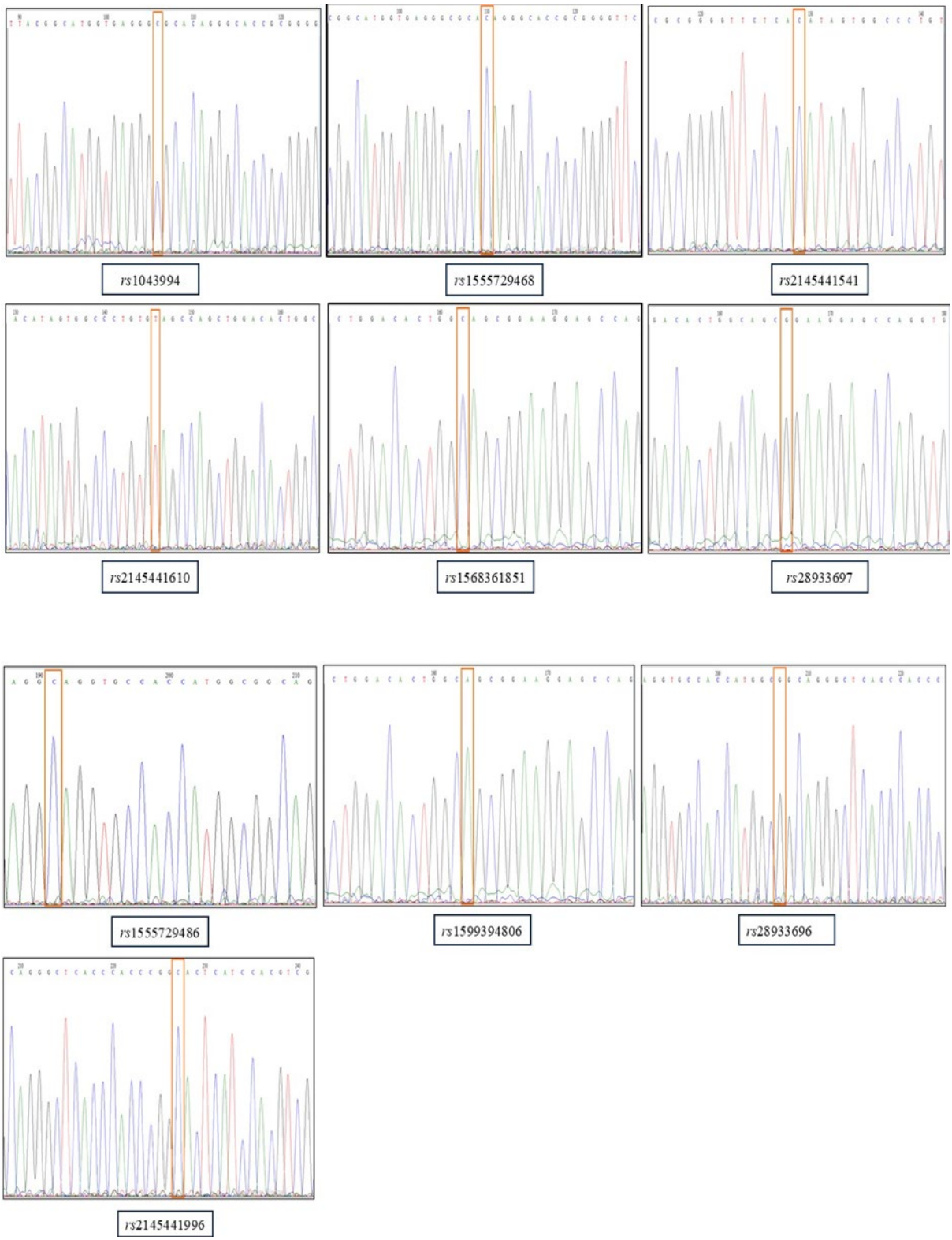

Figure 1. Electropherograms of *NOTCH3* SNPs genotyping by Sanger sequencing

stroke subtype in young individuals, yet its incidence varies significantly due to differences in its definition, diagnostic approaches, and etiological classifications (Divišová et al., 2020; Yaghi et al., 2017). This discussion contextualizes our findings with existing literature and underscores the unique contributions of our study, while highlighting limitations and future research directions.

4. 1 Traditional Vascular Risk Factors

4.1.1 Hypertension

Our findings indicate that hypertension was present in 21.43% of the cohort, aligning with previous studies that emphasize its role as a key risk factor for CIS. Elevated blood pressure has been shown to correlate with reductions in brain volume and increased T2 lesion load, as observed through MRI (Stack & Cole, 2018). Hypertension's contribution to ischemic stroke is well-documented and linked to both large and small vessel disease (Phipps & Cronin, 2020). The association between hypertension and CIS in our study highlights the importance of aggressive cardiovascular risk factor management in young patients.

4.1.2 Smoking

Smoking was identified in 14.29% of the cohort, a finding consistent with prior studies demonstrating its association with a younger onset of ischemic stroke (Schulz, 2019). Smoking exacerbates the risk of acute arterial occlusion through mechanisms such as increased fibrinogen levels, heightened platelet aggregation, and arterial stiffness (Stack & Cole, 2018). The role of smoking in promoting atherosclerosis and vasoconstriction underscores its multifaceted contribution to ischemic stroke pathogenesis. These results reinforce the need for targeted smoking cessation programs tailored to younger populations at risk for CIS.

4.1.3 Obesity

Obesity (BMI > 30) was observed in 11.90% of patients, with a higher prevalence in women (13.79%) than men (7.69%).While the difference was not statistically significant, this trend aligns with studies linking rising BMI to increased stroke risk in young adults (Yuan et al., 2022). Visceral obesity, in particular, promotes thrombosis through chronic low-grade inflammation,
oxidative stress, and endothelial dysfunction (Papakonstantinou et al., 2019). The hormonal differences influencing fat distribution between genders may partially explain the observed variation (Papageorgiou et al., 2024). Our findings emphasize the importance of addressing obesity as a modifiable risk factor in CIS prevention strategies.

4.1.4 Hyperlipidemia

Hyperlipidemia was present in only 4.76% of patients, a relatively low prevalence compared to other studies. The protective role of high-density lipoprotein cholesterol (HDL-C) in reducing endothelial dysfunction and atherosclerotic plaque development has been documented (Wang et al., 2024). Further research is needed to elucidate the relationship between lipid profiles and ischemic stroke in younger populations.

4.1.5 Diabetes Mellitus

Diabetes mellitus was observed in 4.76% of patients, reinforcing its established role as a stroke risk factor. Diabetic patients exhibit endothelial dysfunction, augmented thrombogenesis, and an increased propensity for atherosclerosis and plaque instability (Hu et al., 2021). The association between diabetes mellitus and an elevated risk of myocardial infarction and ischemic stroke underscores the need for comprehensive management of glycemic control and associated cardiovascular risk factors.

4.2 Migraine and Gender Differences

4.2.1 Migraine

In this study, 47.62% of patients reported migraines, with a higher prevalence in women (58.62%) than men (23.08%). Although this difference was not statistically significant, it aligns with research identifying migraine as an independent risk factor for ischemic stroke, particularly in younger adults (Hack et al., 2019). The observed gender disparity may reflect hormonal influences and genetic predispositions, as suggested by recent studies (Mizuta et al., 2024). Further investigation into the pathophysiological mechanisms linking migraines and stroke is warranted.

4.2.2 Stroke Severity and Gender

Women in our cohort exhibited more severe strokes, as evidenced by higher NIHSS scores, and experienced ischemia at a younger age than men. However, no significant gender-specific clinical profiles were observed. Existing literature on gender disparities in stroke severity remains inconclusive, with some studies reporting greater aphasia and visual field deficits in women (Yuan et al., 2022). The underlying reasons for these differences may involve biological, hormonal, and social factors, which merit further exploration.

4.3 Genetic Insights

4.3.1 NOTCH3 Gene and CADASIL Syndrome

The NOTCH3 gene, implicated in CADASIL syndrome, encodes a transmembrane receptor essential for vascular smooth muscle cell function (Papageorgiou et al., 2024). Mutations in exons 2–23, particularly exon 4, disrupt the cysteine residues in epidermal growth factor-like repeats, leading to receptor misfolding and protein aggregation (Papakonstantinou et al., 2019). In our study, genotyping of exon 4 did not identify mutations in the 42 CIS patients examined. This finding suggests that other pathogenic variants in different exons or regions of the NOTCH3 gene may be involved, or that alternative genetic mechanisms underlie CIS in this population.

The absence of mutations does not exclude CADASIL, as clinical diagnosis and MRI findings remain essential for its identification. Mutant NOTCH3 proteins accumulate in the endoplasmic reticulum, causing oxidative stress and impairing cellular homeostasis (Papageorgiou et al., 2024). These effects contribute to

vascular dysfunction, endothelial damage, and the degeneration of the neuro-glio-vascular unit, hallmarks of CADASIL (Mizuta et al., 2024). Further molecular studies are needed to characterize the genetic landscape of CIS and CADASIL in Algerian patients.

4.3.2 Pathophysiological Mechanisms

Our findings align with previous research on the pathological mechanisms underlying CADASIL and other genetic forms of small vessel disease. GOM deposits on microvascular walls impede perivascular fluid drainage, leading to protein accumulation and vascular swelling (Papakonstantinou et al., 2019). Pericyte degeneration and astrocytic end-foot detachment compromise the blood-brain barrier, increasing neurotoxic exposure and neuronal loss (Yuan et al., 2022). These processes collectively disrupt vascular tone, cerebral autoregulation, and neurovascular integrity.

The prolonged retention of mutant NOTCH3 proteins in the endoplasmic reticulum, mediated by chaperone proteins such as calnexin, exacerbates oxidative stress and proteasome dysfunction (Papageorgiou et al., 2024). These events trigger apoptotic pathways, contributing to the progressive degeneration of vascular smooth muscle cells and endothelial cells (Mizuta et al., 2024). Understanding these mechanisms provides a foundation for developing targeted therapies to mitigate vascular dysfunction in CIS and CADASIL.

4.3.3 Clinical Implications

Our study highlights the multifactorial nature of CIS, with traditional vascular risk factors, genetic predispositions, and environmental influences contributing to its pathogenesiss. Identifying and managing modifiable risk factors such as hypertension, smoking, obesity, and diabetes is crucial for preventing ischemic stroke in younger populations. Genetic screening for NOTCH3 mutations and other monogenic causes of small vessel disease should be considered in patients with suggestive clinical and radiological features.

MRI remains an invaluable tool for diagnosing CADASIL and assessing the extent of vascular damage in CIS patients. Advanced imaging techniques, including diffusion-weighted imaging and perfusion studies, can provide additional insights into the pathophysiology of ischemic stroke. Integrating genetic, clinical, and radiological data is essential for accurate diagnosis and personalized treatment strategies.

4.4 Limitations and Future Directions

This study has several limitations. The relatively small sample size may have limited the statistical power to detect significant associations between risk factors and CIS outcomes. The absence of mutations in exon 4 of the NOTCH3 gene does not exclude the possibility of pathogenic variants in other exons or regions. Whole-genome sequencing or targeted next-generation sequencing may provide a more comprehensive analysis of genetic factors in CIS.

The study population's demographic and geographic characteristics may limit the generalizability of the findings to other populations. Future research should include larger, multi-center cohorts to validate our results and explore the genetic and environmental determinants of CIS. Longitudinal studies are needed to assess the long-term outcomes and recurrence rates in young stroke patients.

5. Conclusion

In conclusion, this study provides a preliminary analysis of the clinical presentation and VRFs in young-onset CIS Algerian patients. Although consistent with existing literature, the findings are limited by the small sample size, cross-sectional design, and reliance on self-reported data, which may introduce bias. Genetic analysis of the NOTCH3 gene revealed no mutations among the targeted regions, suggesting the need for further investigation into other exons or novel variants specific to the Algerian population. Larger, more comprehensive studies are essential to validate these results, uncover potential genetic predispositions, and enhance diagnostic and preventive strategies for young-onset CIS in Algeria.

Author contributions

M.L., M.L.R., B.S.F., A.B., B.D., A.I.B., Y.B., and K.S. contributed to the conceptualization, methodology, and investigation of the study. M.L. and M.L.R. supervised the research process. B.S.F. and K.S. handled data analysis and validation. A.B., B.D., and A.I.B. were responsible for data collection and curation. Y.B. and K.S. contributed to the manuscript writing and review. All authors reviewed and approved the final version of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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