UNIVERSITATEA LUCIAN BLAGA DIN SIBIU

Doctoral School of Medicine

Doctoral field: Medicine

ABSTRACT OF DOCTORAL THESIS

Contributions in studying the role of inflammation, coagulation and atherogenesis in acute ischemic stroke pathogenesis.

Ph.D. candidate
Maria-Gabriela Catană (Vlădoiu)

Ph.D. supervisor:
Prof. Univ. Dr. Romeo-Gabriel Mihăilă

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Reason for choosing the research subject

The American Stroke Association defines stroke as an episode of neurological dysfunction caused by a focal cerebral, spinal, or retinal infarction. Stroke annually produces 9% of all deaths worldwide, being also one of the important causes of disability (1). The most common cause of cerebrovascular accidents is the occlusion of blood vessels at cerebral level, through thrombosis or embolism (85% of cerebrovascular accidents are ischemic) (1). Contrary to all the years of study, therapeutic options for stroke are limited, with most patients receiving only supportive care. Recent studies show that stroke recurrence can occur in 12-20% of patients within the first 90 days (2). Among the multiple risk factors of vascular accident are: arterial hypertension, atrial fibrillation, diabetes, dyslipidemia, atherosclerosis, diseases with thrombogenic potential - thrombophilia, obesity. Atherosclerotic disease is one of the main risk factors of vascular accident. Studies show that 5% of women and 12% of men have asymptomatic moderate carotid stenosis (carotid diameter reduced between 50% and 70%) (2).

In the present paper I aimed to study the role of inflammation generated by the occurrence of an ischemic stroke and its role in terms of both atherosclerotic disease and the prognosis of these patients. The present work is original in that at the end of it I propose to compile a risk score for determining the severity and prognosis in patients who have suffered cerebral ischemia. This score should be of real use in the day-to-day practice of clinicians in neurology departments and not only who encounter this pathology so often.

Keywords: ischemic stroke, inflammation, interleukin 6, interleukin 1β, tumor necrosis factor α, SARS-COV2, thyroid hormones, atheromatosis, subclinical hypothyroidism, subclinical hyperthyroidism.

Introduction

Part I – The current state of knowledge

Stroke is defined as an episode of neurological dysfunction caused by a focal cerebral, spinal, or retinal infarction. Stroke annually produces 9% of all deaths worldwide, being also one of the important causes of disability [1].

Ischemic stroke, which is caused by the obstruction of a blood vessel by a thrombus, accounts for 87% of all strokes [1].

After years of extensive research and despite advances in the understanding of stroke pathophysiology, therapeutic options for stroke remain very limited. Currently, the only approved therapy for the treatment of acute cerebral ischemia is the intravenous administration of recombinant tissue plasminogen activator (rt-PA) together with endovascular mechanical thrombectomy to remove the thrombus [2]. However, treatment with rt-PA carries a significant risk of bleeding. In addition, the window of action has a short time and is administered within 4.5 hours of the onset of symptoms. This limitation reduces to approximately 15% the number of stroke patients who can be treated. Although mechanical thrombectomy has recently demonstrated its efficacy even 24 hours after the onset of the cerebrovascular event, neuroprotective therapies are sought to save the compromised tissue in the peri-infarct area of the brain [3].

Both hemorrhagic and ischemic stroke lead to permanent infarction of the neurons located at the base and center of the affected region. The area surrounding the infarcted side contains oligoemic neurons. This makes the ischemic penumbra a promising target for possible stroke therapies. Evidence accumulated over years of studies supports the fact that inflammation has a key role in the pathophysiology of atherosclerotic plaque and its destabilization. Studies indicate the involvement of inflammatory cells in all stages of the development of atherosclerosis. Circulating monocytes migrate transendothelially initiating atherosclerotic plaque formation in response to external risk factors such as blood pressure, non-laminar flow, cigarette smoke constituents and angiotensin 2 [4]. Retention of low-density lipoproteins (LDL) in the extracellular matrix of the arterial wall is accompanied by differentiation of monocytes and macrophages. The differentiation of macrophages is carried out in subgroups, namely pro-inflammatory macrophages (M1 macrophages), with a role in the local expression of pro-inflammatory cytokines, in particular interleukin 1-beta (IL-1 β) and tumor necrosis factor-alpha (TNF-α) .

Cytokines such as platelet-derived growth factor are involved in the recruitment and proliferation of smooth muscle cells in the endothelial cell layer, which express matrix proteins such as collagen and elastin [5].

Almost all components of the neurovascular unit, including endothelial cells, pericytes, neurons, and glial cells, are involved in the development of neurovascular injury. Endothelial cells form a dynamic interface between the blood-brain barrier and peripheral tissues, which are essential for maintaining neurovascular homeostasis. Consequently, endothelial cell injury is considered one of the first symptoms of cerebral ischemia. Pericytes were initially recognized as a contractile cell type with a role in regulating neurovascular tone, but were later found to also respond to stress-related injury caused by brain pathology. The loss of normal glial cells can contribute to reduced support for neurons and the emergence of dysfunctions at the level of the neurovascular unit. Astrocytes and microglia are the primary cells responsible for the immune system in the central nervous system with a role in controlling pathogenic invasion. Neuroglia also produces signals to activate and recruit cells that contribute to adaptive immunity to ultimately clear the infection. Cerebral atrophy is also a significant factor affecting the integrity of the neurovascular unit [3-6].

Stroke is the second leading cause of death globally. It affects about 13.7 million people and kills about 5.5 million annually. About 87% of strokes are ischemic infarcts, a prevalence that has increased substantially in recent years. The incidence of stroke doubled in low- and middle-income countries, but decreased by more than 42% in high-income countries [7].

The incidence of stroke increases with age, doubling after age 55. However, in an alarming trend, strokes in people aged 20 to 54 have risen from 12.9% to 18.6% globally. However, age-standardized attributable death rates decreased by 36.2% during the same period [5–7]. The highest reported incidence of stroke is in China, where it affects approximately 331–378 individuals per 100,000 life years. The second highest rate is in Eastern Europe (181–218 per 100,000 life years) and the lowest in Latin America (85–100 per 100,000 life years) [8].

The occurrence of stroke in men and women also depends on age. The incidence is higher at younger ages in women, while it increases slightly with age in men. The high risk of stroke in women is due to pregnancy-related factors such as preeclampsia, contraceptive use and hormone therapy, as well as migraine with aura [9]. Atrial fibrillation increases the risk of stroke in women over 75 by 20%. Based on the NIHSS (National Institute of Health Stroke Scale) for stroke (0 = no stroke, 1–4 = minor stroke, 5–15 = moderate stroke, 15–20 = moderate/severe stroke, 21–42 = severe stroke), mean stroke severity was estimated at 10 points for women and 8.2 points for men. Both cerebral infarction and intracerebral hemorrhage (ICH) are common in men, but cardioembolic stroke is more prevalent in women. The mortality rate for stroke is also higher among women [5–7]. For men, the most common causes of stroke are tobacco smoking, excessive alcohol consumption, myocardial infarction and arterial pathologies [9].

A recent study examining the prevalence of stroke and related risks examined demographics, behavior, physical characteristics, medical history, and laboratory tests and revealed the contribution

of air and particulate pollution exposure to stroke mortality [10]. Another study found that hypertension is a statistically significant risk for stroke, especially ischemic stroke [11]. A study in the United States (US) also identified hypertension as a major cause of stroke and described geographic variations in the severity of ischemic stroke symptoms. Insufficient physical activity, poor dietary habits, and consumption of nicotine and alcohol were considered additional risks [12]. Differences in exposure to environmental pollutants such as lead and cadmium also influenced the incidence of stroke in different regions. This study also revealed differences in the incidence of stroke between non-Hispanic black and white populations aged 40–50 years [13].

There is a strong inverse relationship between stroke and patients' socioeconomic status, which is due to inadequate hospital facilities and post-stroke care among low-income people [14]. A US case study showed that people with high financial status had better stroke treatment options than disadvantaged people [15]. Research conducted in Austria associated education level with adoption of paraclinical treatments or examinations such as echocardiography and speech therapy; however, there was no difference in the provision of thrombolysis, occupational therapy, physiotherapy or secondary stroke care by socioeconomic status [17]. Similarly, in the Scottish healthcare system, basic treatments such as thrombolysis were provided regardless of patients' economic status [18].

The current gold standard in stroke assessment is cerebral and neurovascular imaging, in addition to clinical assessment of stroke severity using the NIHSS scale. Non-contrast computed tomography of the brain is rapid, widely available and cost-effective. It is used as primary imaging in patients with suspected acute ischemic stroke to primarily rule out an acute hemorrhage [19]. Non-contrast CT images are widely used to assess the location and extent of stroke using the Alberta Stroke Early Score CT (ASPECTS) program [20]. Expert interpretation of CT images can diagnose major stroke but is significantly insensitive in diagnosing minor stroke. Computed tomography also has low sensitivity (<20%) in the first 3 hours after the onset of cerebral ischemia and 57–71% sensitivity at 24 hours [21].

CT angiography (CTA) and perfusion computed tomography (CTP) are routinely used for diagnosis and selection of patients for endovascular therapy (EVT) [22]. The major disadvantage of this method is the time required to acquire the sequential images, but the advancement of automated software makes it easier to reduce the time. While imaging time is increased with CTP/CTA, one study showed that total treatment time was not increased with CTA and CTP compared with native cranial CT alone, likely due to rapid evaluation and better anatomic data before by EVT [23].

Single-phase CT angiography (sCTA) is useful for rapid evaluation of large vessel occlusions and is also useful for evaluating collateral circulation. Good collaterals on sCTA correlate with reduced increase in cerebral ischemia, although no correlation was observed between collateral status and patient clinical status [24]. CTA immediately followed by native CT is recommended for all

ischemic stroke presentations [18]. Many endarterectomy studies used CT and CTA for patient selection and used ASPECTS to estimate infarct extent. Comparison of sCTA data from different studies can be problematic due to inconsistency in the timing of contrast injection and image acquisition [19]. In addition, various collateral circulation scoring methods for sCTA have been suggested, but there is no accepted standard of this assessment method [20].

Multiphasic CTA (mCTA) provides three time-resolved grayscale images of the cerebral vasculature with delays of 8 and 16 s. Thus, the reader must stitch these images together for viewing and interpretation, which requires a high degree of expertise. mCTA provides a consistent assessment of pial arteries in addition to images of cerebral infarction and has good inter-rater reliability as well as predictability of clinical status with remarkable accuracy [21]. mCTA is more accurate in detecting distal vessel occlusion than non-complementary sCTA [20,21].

Perfusion CT (CTP) provides comparable diagnosis and prognosis to mCTA and is often preferred because the color maps used are easier to interpret [25]. CTP also uses images of parenchymal blood flow and a single color-coded cerebral blood flow map designed to display a likely tissue prognosis using sophisticated acquisition and post-processing tools. CTP assesses blood flow in capillary tissue using data from repeated brain scans. Perfusion parameters used in identifying the ischemic core and penumbra include cerebral blood flow (CBF), cerebral blood volume (CBV), mean transit time (MTT), time to peak (TTP), and time to peak (Tmax). Although there is no clear consensus in the literature regarding the exact parameters or thresholds that should be used to characterize the core and penumbra, brain tissue with severely reduced CBV or CBF predicts infarction of the core and collateral regions. Prolongation of MTT or its derivatives, TTP or Tmax, causes penumbra [23, 25].

Digital subtraction angiography (DSA) is an old technology that requires invasive action and is not widely used today. DSA can also be used to determine the location of the occlusion and assess collateral circulation, most commonly during EVT [26].

Magnetic resonance imaging (MRI) has a higher sensitivity than any other imaging techniques for detecting ischemic stroke. However, it is not as available as other imaging modalities and it is mainly used as follow-up imaging [26]. Diffusion MRI or diffusion-weighted imaging (DWI) is the gold standard for the imaging diagnosis of cerebral ischemia, with its detection possible as early as 30 minutes after the onset of symptoms. In DWI, proton diffusion in tissue is shown, so tissues with delayed or restricted proton movement with an apparent decrease in diffusion coefficient (ADC) appear bright. While native CT, angio-CT and perfusion CT can be used to estimate or infer the size of the infarcted core and penumbra, DWI provides more sensitive and specific measurements of the volume of acutely infarcted brain tissue [26, 27] .

Magnetic resonance angiography (MRA) can be used as an alternative tool to CTA to assess brain perfusion, although it is not as widely used as CTA. Both sCTA and MRA enable rapid detection of large vessel occlusion [26]. Overall, the performance and imaging data readout of sCTA and MRA are technically no more difficult than perfusion imaging and are effective for treatment decisions regarding ischemic stroke [27].

The 2018 guidelines for the management of acute cerebral ischemia from the American Heart Association/American Stroke Association now recommend that CTP imaging, diffusion weighted MRI (DWI) - MRI be included as part of a standard imaging evaluation for patients who are within 6 -24 hours from the onset of symptoms [28].

A panel of accurate blood biomarkers could be an important additional diagnostic tool [30]. Characteristics for ideal blood biomarkers for stroke should primarily have a high level of specificity and sensitivity similar to characteristics of imaging biomarkers. This is essential for differentiating strokes from stroke-mimics and for differentiating between hemorrhagic and ischemic stroke. Ideal biomarkers should be involved in cellular processes that are unique to ischemic stroke, which is very challenging [31,32].

Given the complexity of stroke, different mechanisms are believed to be involved in its pathophysiology. In fact, growing evidence shows that inflammation plays an important role in the progression and prognosis of ischemic stroke. For this reason, therapeutic targeting of postischemic inflammation in acute stroke has gained interest as a potential neuroprotective strategy [51].

Recent data in the literature emphasize a certain relationship between inflammation and ischemic type lesions of the central nervous system. This acute neuroinflammatory process involves movements of microglia resulting in morphological and phenotypic changes along with the release of inflammatory mediators such as chemokines and cytokines [52].

Even if inflammation at the cerebrovascular level cannot usually be considered an initiating factor of neurodegenerative disorders, it represents a balance between pro- and anti-inflammatory cytokines that determine sustained inflammatory responses at the level of the central nervous system in terms of lesion progression. Activated microglial cells present a variety of proinflammatory cytokines such as TNF-alpha, IL-6, and IL-1, as well as nitric oxide (NO) and superoxide, which are neurotoxic and may enhance the processes underlying ischemic injury. Although there are several causes and several risk factors have been identified for various neurological disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) it has been clearly established and a active role of cerebrovascular inflammation. Activation of innate immune responses can be mediated by the engagement of specific pattern recognition receptors (PRRs), such as TLRs, to initiate pro-inflammatory responses and activate

adaptive immunity. The ultimate outcome of brain diseases will be based on the role of inflammation and the alteration of the inflammatory state determined by vascular risk factors [52, 53].

Overt clinical hyperthyroidism predisposes patients to atrial fibrillation, a cardiovascular pathology that can produce cerebral ischemia through a cardioembolic mechanism. On the other hand, hyperthyroidism can be associated with an increase in other risk factors for cerebral infarction, such as atherosclerosis and dyslipidemia [97]. Studies show, however, that this hypothesis has not been clearly elucidated, a direct relationship between the values of thyroid hormones – FT4 (thyroxine), TSH (thyrotropin), the severity and sequelae of cerebral infarction not being clearly established [98]. Hyperthyroidism is associated with a hypercoagulability status, by increasing the level of coagulation factors – fibrinogen, FVIII, FIX, FX, von Willebrand factor. At the same time, hyperthyroidism causes hypofibrinolysis by reducing plasmin and plasmin activator [98]. Recent studies have shown that chronic hyperthyroidism aggravates cerebral injury caused by cerebral infarction, and also that subclinical hyperthyroidism (low TSH, FT4 within reference limits) negatively affects functional recovery after ischemic stroke. Hyperthyroidism increases the risk and mortality caused by acute cardiovascular events, but further studies are needed to concretely prove the association of this endocrine pathology with cerebrovascular diseases [99].

Several studies have found an association between thyroid dysfunction and the prognosis of patients with acute ischemic stroke in recent years. Essentially, thyroid hormones drop shortly after the onset of stroke symptoms. They decrease in the first days, then in the chronic stage they reach normal limits, while TSH continues to decrease. Moreover, severe strokes probably lead to a stronger decrease in fT3, which ultimately results in worse clinical prognoses and increased mortality, as reported by several researchers [100].

Currently, the results of studies on the relationship between thyroid-stimulating hormone (TSH) and patients with acute ischemic stroke are controversial. Some studies have reported the protective effects of elevated TSH on severity and prognosis in patients with acute ischemic stroke [100]. Subclinical hyperthyroidism is associated with poor prognosis in patients with acute ischemic stroke. Patients with subclinical hypothyroidism tend to have milder strokes and a favorable functional outcome. Conversely, a study in patients with acute cerebral ischemia reported that TSH levels in patients with severe ischemic stroke were higher than those in patients with mild or moderate stroke, and that TSH levels were higher in the group with less recovery obvious [101].

As the most sensitive index for evaluating thyroid function, TSH also interacts with thyroid hormones. Unfortunately, few studies have ruled out the potential influences of thyroid hormone changes when exploring the association between TSH and stroke. Furthermore, the mechanism by which TSH affects stroke is still unclear. Many studies have found that TSH levels are associated with serum lipid profiles in healthy individuals, and the association still exists in patients with

coronary heart disease and type 2 diabetes [102]. In the acute phase of COVID-19, TSH levels are also associated with reductions in total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C). However, the association between TSH and lipid profiles has not been evaluated in patients with acute ischemic stroke [102].

The potential mechanism by which TSH is associated with acute ischemic stroke is not well understood. Several hypotheses have been put forward to explain the favorable effect of elevated TSH on stroke severity. One possible mechanism is hypometabolism. It is well known that higher TSH levels lead to a decrease in basal metabolic rate. The reduced response to physical stress may also explain the reduced severity in patients with higher TSH levels. A decrease in sensitivity to adrenergic stimulation has been observed in patients with hypothyroidism [103]. Another possible explanation is that higher TSH levels may increase ischemic tolerance, which is based on a theory that sublethal ischemia renders affected tissues resistant to subsequent, more severe ischemic attacks.

Several studies have provided evidence that subclinical hypothyroidism could increase the risk of atherosclerosis by increasing systemic vascular resistance, arterial stiffness, endothelial function, lipid abnormalities, and insulin resistance [104]. Therefore, a higher level of TSH induces atherosclerosis and a neuroprotective mechanism of ischemic preconditioning is initiated. A number of studies have reported that a high TSH level is associated with lipid metabolism and cardiovascular risk. The association between TSH and lipid profiles has also been confirmed in coronary artery disease and type 2 diabetes, but has not been studied in acute ischemic stroke. Possible explanations for the effects of TSH on the lipid profile have not been fully elucidated. Studies have shown that liver cells express the TSH receptor. One study found that TSH can act on the TSH receptor in hepatocyte membranes and promote the expression of hepatic 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, a rate-limiting enzyme in cholesterol synthesis, thereby increasing synthesis and raising cholesterol levels [105].

TSH can also act on TSH receptors expressed on adipocytes, thereby greatly increasing adipogenesis and lipolysis, which can also increase serum free fatty acid levels. Insulin resistance may also be the mechanism by which TSH affects lipid profiles [106]. The direct effects of TSH on lipids require further investigation.

Contrary to the different mechanisms of primary injury, inflammation is triggered by a common pathway for both acute ischemic stroke and intracerebral hemorrhage and plays a crucial role in the development of cerebral edema and other pathophysiological consequences. Furthermore, the evidence reviewed from recent research presents a complex picture of inflammatory responses to brain injury that are not limited to the site of injury, but may occur globally and persist long-term. Global brain inflammation can persistently shape the pathophysiology of brain injury after stroke and promote the decline of global brain functions such as cognition. Future research should not only aim

to understand how global brain inflammation is initiated and maintained, but also to clarify the role of inflammation in the long-term sequelae of stroke and its roles in neurological recovery and brain tissue regeneration [107].

These efforts would pave the way for the design of immunomodulatory therapies to reduce inflammation associated with brain injury.

Recognition of the crucial role of inflammation after stroke has inspired various clinical trials aimed at ameliorating brain inflammation in an effort to counteract secondary brain damage in the acute and subacute stages of ischemic stroke. Although with limited sample sizes in some studies, these trials have yielded encouraging results, and include evaluation of natalizumab in acute ischemic stroke, fingolimod in acute ischemic stroke89 and intracerebral hemorrhage, and glyburide in cerebral infarction [108]. Several new large-scale clinical trials based on insights derived from these studies are ongoing (NCT02730455, NCT02864953, NCT03338998, and NCT02956200). In addition, new genes and molecules responsible for the development of inflammation are identified from profiling relevant tissue, such as peri-hematomal tissue derived from patients with intracerebral hemorrhage. The question is whether these genes and molecules can serve as drug targets and whether there is a so-called master switch that orchestrates the inflammation cascade [106-108].

Resolving these issues would be essential in designing future therapies for stroke patients. The overriding question is whether a better understanding of cerebral neuroinflammation would lead to successful clinical translation of immunomodulators for stroke. Previous efforts to manipulate the immune system have involved targeting adhesion molecules (ie, natalizumab), cell efflux (ie, fingolimod), or cytokines in the periphery. These approaches prevent the development of neuroinflammation but do not alter ongoing in situ processes. Direct interference with molecules that trigger local brain inflammation is likely to reduce the expansion of brain lesions [109].

Admittedly, we are still far from fully understanding the role of inflammation and immune responses in ischemic stroke. Several studies have described inflammatory and immune responses after stroke in various experimental models (Gelderblom et al., 2009; Zhou et al., 2013; Drieu et al., 2020a, 2020b) and humans and it is undeniable that neuroinflammation and immune responses are present minutes after stroke onset and last for months or even years [110]. Moreover, anti-inflammatory and immunomodulatory therapies have shown beneficial effects in some of the experimental models. Some of these molecules have been tested in randomized clinical trials, but have not had positive results in stroke patients. This fact suggests that the mechanisms by which inflammatory processes contribute to neuronal injury after stroke are not sufficiently elucidated and could reflect a mismatch between the inflammation produced in these particular experimental models and in stroke patients [111].

The development of multicenter preclinical studies would help to confirm positive preclinical results before proceeding to clinical trials. Moreover, comorbidities (inflammatory conditions, previous stroke, etc.) are often neglected in experimental studies and thus far from the clinical reality of stroke patients.

The choice of clinically relevant experimental models of stroke, the inclusion of measurements including acute but also long-term quantification of infarct volume and behavioral prognosis, the performance of multicenter studies and the inclusion of coexisting risk factors are mandatory before considering consider translating a new therapy for ischemic stroke into randomized clinical trials.

Atherosclerosis has become an important neurological and cardiovascular pathology in the last decade, being a frequent cause of mortality and morbidity. Atherosclerotic plaque formation is a dynamic process that extends to all arteries throughout the body. This process is influenced by both genetic and environmental factors, as well as specific medication [112].

Hypercholesterolemia, hypertension, smoking, diabetes and obesity are risk factors for the development of atherosclerotic plaque, and inflammation is the final pathway for these risk factors as well as an independent factor of atherogenesis [112].

The progression of atheroma plaque is influenced by the release of pro-inflammatory cytokines, thus demonstrating the causal relationship between inflammation and atherogenesis. Inflammation is basically a complex beneficial response of the host to exposure to microbial invasion, tissue damage, or other stimuli, helping to eliminate the initial cause of cellular injury. However, inflammation is also a major cause of cardiovascular disease, mainly when an imbalance of homeostasis occurs - the inflammatory response being too strong or too long (intractable inflammation). The concept of atherosclerosis as a chronic inflammatory condition is no longer controversial, but the understanding of immune regulation is still incomplete [113].

Carotid artery stenoses are caused by thickening of the arterial wall due to atheromatosis that occurs preferentially at the bifurcation of the carotid artery or at the emergence of the internal carotid artery from the common carotid artery. Carotid artery stenoses occur in approximately 7% - 9% of the population, usually being associated with neurological pathology - ischemic stroke or cardiovascular pathology - coronary artery disease [113]. The progression of atheromatosis at the level of the carotid arteries is intensified by certain risk factors such as: dyslipidemia, arterial hypertension, diabetes, smoking and hemodynamic disorders at the level of the vessel - for example, turbulent blood flow. The most recent guidelines recommend surgical intervention (vessel endarterectomy) in >70% stenoses within the first two weeks after the onset of a minor ischemic stroke or transient ischemic attack [114]. The same international guidelines do not recommend surgical intervention in asymptomatic carotid stenoses. A recent prospective study demonstrated that

asymptomatic carotid artery stenoses >50% are associated with an annual ischemic stroke rate <1% [115].

Ischemic vascular events may be caused by atherosclerotic lesions that may enlarge or ulcerate leading to the formation of intraplaque thrombi and thus causing emboli. The composition of the atheroma plaque is a very important feature because it can dictate its instability, along with its size and location. The necrotic center is one of the characteristics of unstable plaques because liquid cholesterol can accumulate in it, which can lead to ulceration of the plaque. Cholesterol crystals that form at the level of the ulcerated atheroma plaque stimulate the secretion of interleukin 1 Beta (IL-1β), thus triggering the inflammatory response and as a consequence increasing the risk of acute events [116].

Inflammation is the basis of atheromatosis and greatly influences its presence and development. Patients with atherosclerosis have elevated levels of inflammatory markers, associated with disease severity and clinical outcome. The cause of persistent inflammation during atherogenesis is not fully understood. Today, a Western diet, smoking, and a sedentary lifestyle, which in turn lead to hyperlipidemia and obesity, are the most important drivers of atherosclerotic plaque, with several studies suggesting that inflammation is a common endpoint for these risk factors [123]. However, atherosclerosis has also been described in CT images of mummies dating back 4000 years, demonstrating that atherosclerosis is not just a modern condition [121].

Studies show that in mice, hypercholesterolemia stimulates neutrophilia, with subsequent early lesion formation. Chemotactic stimuli recruit neutrophils to atherosclerotic plaques, accumulating especially in regions prone to rupture. Neutrophils stimulate monocyte recruitment by releasing granule proteins, as well as monocyte trapping and dendritic cell activation in the plaque by forming neutrophil extracellular traps (NETs). NET formation, or NETosis, is a specialized form of cell death involving ROS production, histone modification, and chromatin release. NETs released from activated neutrophils capture invading microorganisms, preventing their spread [126].

While quantification of cytokines and other inflammatory markers may be useful when assessing the degree of inflammation in patients with atherosclerosis, they do not reflect inflammation within the atherosclerotic lesion. In recent years, several studies have investigated the development of diagnostic modalities that can assess plaque inflammation. Therefore, intravascular ultrasonography allows direct visualization of atheroma in vivo including characterization of vascular remodeling. Moreover, positron emission computed tomography (PET-CT) can directly assess plaque inflammation, especially when combined with nanoparticles that can demonstrate macrophage activation and endothelial activation [125, 126].

Increased knowledge of immune regulation in atherosclerosis also opens the door to developing a vaccine to prevent atherosclerosis. Both direct and indirect vaccinations have been suggested. For

direct vaccinations, immunization with LDL or modified LDL components has shown promise in animal models [127].

One of the most indirect approaches is the flu shot. An increase in the incidence of acute cardiovascular events is seen during the flu season. The mechanisms by which the influenza virus increases the risk of cardiovascular disease are not known. Thus, vaccination may also have potential in the secondary prevention of CVD and highlights the need for further studies on the mechanisms underlying these observed results [127].

CANTOS is a randomized, double-blind trial in which 10,061 patients with previous myocardial infarction and elevated levels of inflammation were included. High-sensitivity C-reactive protein (hsCRP) (> 2 mg/L) was used as a marker. Overall, 80% of study participants were treated for hypertension, 40% had diabetes, and >90% were on statin therapy. They were randomized into 4 groups to receive canakinumab, a human monoclonal antibody against the inflammatory cytokine, interleukin(IL)-1β at 3 different doses, or placebo. Canakinumab is currently used clinically in some rare autoinflammatory conditions and as a second-line treatment in rheumatoid diseases. As expected, canakinumab reduced high-sensitivity C-reactive protein levels, but without a substantial effect on plasma lipids [132].

The main objective of the study was to stop the occurrence of myocardial infarction, stroke or sudden cardiovascular death among the patients included in the study. Canakinumab significantly reduced this end point, from 3.9 years per 100 people in the placebo group to 4.5 in the groups receiving ≥150 mg of antibody per dose. This translated into a 15% relative risk reduction. The effect was similar in those who received 300 mg per dose, while the group who received 50 mg per dose obtained no benefit. This finding suggests that the beneficial threshold to achieve protection is somewhere between 50 and 150 mg [132].

Serious side effects were rare and mainly consisted of infections. However, the simple fact that fatal infections were higher in the active treatment groups (0.31% versus 0.18% in the placebo group) calls attention to careful monitoring of infections in future trials involving canakinumab [132].

The results of the CANTOS study provide strong support for the hypothesis that atherosclerosis is an inflammatory disease. The process is triggered when low-density lipoproteins accumulate in areas of disturbed flow in the blood vessel, and therefore it is believed that hypercholesterolemia and hypertension act on the initiation of atherosclerosis. Pathologically, the resulting process is one of chronic inflammation, accompanied by repair processes and ultimately thrombosis. Briefly, atherosclerosis is an inflammatory disease caused by the accumulation of cholesterol in the arteries [132].

Part II – Personal contribution

Primary and secondary objectives of the research

The main objective – evaluation of the correlation between serum inflammatory markers – interleukin 6, interleukin 1 Beta, TNF alpha and the prognosis and risk of cerebral reinfarction in certain categories of patients (patients without ischemic stroke in the personal pathological history).

Secondary Objectives:

1. Determination of high-sensitivity C-reactive protein and establishing a correlation between it and the severity of ischemic stroke

2. Determination of leukocytes and erythrocyte sedimentation rate in patients with stroke, establishing a correlation between them in order to be able to meet a possible worsening of patients who have suffered an ischemic stroke.

3. Establishing correlations between inflammation biomarkers and the risk of death in patients who have suffered an ischemic stroke, co-infected or not with the SARS-COV2 virus.

4. Assessment of the degree of narrowing of the lumen of the internal carotid arteries, as well as the presence and type of atheroma plaques in patients who have suffered an ischemic stroke.

5. Establishing the risk factors and comorbidities of patients who have suffered an ischemic stroke, highlighting the main comorbidities that could contribute to a new recurrence.

6. Establishing the correlation, if any, between thyroid hormones in patients who have suffered an ischemic stroke and with previously undiagnosed thyroid pathology, with cholesterol and the degree of narrowing of the lumen of the internal carotid arteries.

7. Analysis of the quality of life of patients who have already suffered a stroke through certain scales (Rankin). Assessing the impact of such an event on the ability to carry out daily activities.

8. Compilation of a risk and prognostic score of the vascular accident with real applicability, following the corroboration of the accumulated data.

General research methodology. Ethical considerations.

The research was carried out between January 2020 and December 2021 and was approved by the ethics board (approval attached to this paper). In all the studies carried out in this research, patients with neurological pathology, namely ischemic stroke, who were hospitalized in the Neurology clinic of the Sibiu County Emergency Clinical Hospital, were included. All studies were conducted on the basis of a protocol according to national and international requirements regarding medical research on human subjects and strictly followed the principles stipulated in the Declaration of Helsinki. All patients included in the studies in this research signed an informed consent (attached to this paper). Prior to signing the informed consent, all patients were informed about the terms of conducting the studies, the conditions of withdrawal from the study and, at the same time, they were assured of the confidentiality of the data provided.

For each study included in this research, a research protocol was established and certain steps were followed, as follows:

1. Inclusion of patients in the research groups, according to the inclusion criteria established for each individual study.

2. Establishing the diagnosis of ischemic stroke with certainty (exclusion of stroke mimics) and the onset of symptoms (< 24 hours).

3. Establishing demographic data, personal pathological antecedents of the patients included in the study (data obtained through anamnesis or from the observation sheet) and confirmation/denial of infection with the SARS-COV 2 virus (for study 2).

4. Quantification of ischemic stroke severity using the NIHSS scale. The NIHSS scale was performed dynamically.

5. Collecting in the first 24 hours (and at 7 days for study 2) biological samples and performing extracranial doppler ultrasound of the carotid arteries.

The specific methodology as well as the specific inclusion/exclusion criteria are presented in detail within each study conducted.

Statistical analysis

The database was created using the Microsoft Office Excel 2016 application. The SPSS 25.0 program (SPSS Inc, Chicago, USA) was used for statistical analysis and data description. With the help of the Shapiro-Wilk or Kolmogorov-Smirnov tests, the normality of the distribution of the quantitative data was checked. An error threshold of 0.05 (α =0.05) was accepted.

Arithmetic mean ± standard deviation was used to describe normally distributed continuous quantitative data and median (quartile 1-quartile 3) was used for data that did not have a Gaussian distribution. Qualitative data were described using frequencies.

The Student's test (t-test) was used to compare the means of the corresponding quantitative variables of two independent groups if the variables were normally distributed. To compare the means of two independent groups, in which the variables had an abnormal distribution, the non-parametric Mann – Whithney and Kruskall – Wallis tests were used.

The correlation analysis was done using the Pearson linear correlation coefficient for data with normal distribution, respectively the Spearman correlation coefficient for quantitative data without normal distribution or for ordinal data. Colton's empirical rules were used to interpret the correlation coefficients.

To determine the diagnostic value of some parameters, ROC (Receiver Operating Characteristic) curves were constructed and compared. The graphical relationship between Sn and Sp for the possible threshold (cut-off) values is illustrated with the help of the ROC curve. The optimal values from the point of view of the reliability of the analyzed parameters are considered cut-off points. For each variable considered, sensitivity is represented on the abscissa, and 1-specificity (rate of false positive tests) on the ordinate.

If the area under the curve AUC (Area Under the Curve) is greater than 0.8 (80%), a variable is considered satisfactory as a diagnostic criterion. The closer the AUC value is to 1 (AUC=1 corresponds to the particular situation where the ROC curve reaches the upper-left corner of the figure), the better the diagnostic accuracy of the considered variable.

STUDY 1 – Evaluation of standard acute phase reactants (hsPCR, neutrophil/lymphocyte ratio – NEU/LYM, platelet/lymphocyte ratio – PLT/LYM) in inflammation caused by ischemic stroke

1.1 Introduction

Up to now, updated reliable markers for the prognosis of ischemic stroke are: imaging (computed tomography and MRI) and NIHSS (National Institute of Health Stroke Scale) score [143,144]. Numerous studies have questioned the role of inflammation in ischemic stroke, highlighting the need for biomarkers in order to establish the prognosis of patients with neurological vascular pathology.

Over time, research in the field has confirmed that the inflammatory response aggravates cerebral ischemic lesions [144-146], it being known that leukocytosis noted at the time of hospitalization can be associated with the severity of the stroke and unfavorable prognosis in terms of patients with acute ischemic stroke (AIS) [147].

Neutrophil-lymphocyte ratio (NEU/LYM) and platelet-lymphocyte ratio (PLT/LYM) have been included in recent research as new and inexpensive biomarkers of inflammation studying their diagnostic and predictive capacity in multiple pathologies (including stroke) [148-150]. Many recent studies have considered neutrophil counts and low lymphocyte counts as correlates of adverse functional outcomes in patients who have suffered acute cerebral infarctions (Zhang et al., Xue et al.) [151-153].

Regarding platelets, the information is not sufficiently elucidated because the link between increased platelet count and clinical prognosis remains uncertain. Until now it is known that platelets have an essential role in thrombogenesis and inflammation [154-156].

Therefore, the role of inflammation in ischemic stroke is still insufficiently elucidated, which is why we need more studies that include biomarkers that may be of real benefit to patients with ischemic stroke.

The purpose of the study

The specific objectives of the study

• evaluation of the correlation between serum inflammatory markers - hsCRP, neutrophils, lymphocytes, platelets (standard acute phase reactants) and the severity of ischemic stroke, as well as the prognosis of patients with this pathology.

• calculating the ratio of neutrophils/lymphocytes and platelets/lymphocytes in order to establish a possible correlation between them and the severity of the cerebral infarction, respectively the degree of carotid stenosis.

1.2 Material and methods

The research group included adult patients who suffered an ischemic stroke, with an onset of no more than 24 hours and who were admitted to the Sibiu County Emergency Clinical Hospital between January 2020 and June 2020. Patients included in the study were selected consecutively from the Neurology Department of the Sibiu County Emergency Clinical Hospital and met all the inclusion and exclusion criteria.

The patients were divided into two groups:

• A RESEARCH GROUP: Consisting of 100 patients who suffered an acute ischemic stroke (patients presented to the emergency department within the first 24 hours from the onset of symptoms).

• A CONTROL GROUP: Consisting of 50 patients with no personal pathological history of ischemic stroke, of age and gender comparable to the research group.

Data collected retrospectively and at the time of inclusion in the study: age, gender, PPA (associated conditions), biological: HLG – the ratio of neutrophils – lymphocytes, platelets – lymphocytes is calculated.

The neurological examination was performed and the NIHSS score was calculated, which shows the severity of the vascular accident (affecting the infarcted territory) in dynamics.

Paraclinical: carotid doppler ultrasound - quantification of carotid stenoses in percentages.

The two groups were compared in terms of inflammatory markers (NEU/LYM and PLT/LYM ratios) and in terms of carotid Doppler ultrasound (carotid stenosis) to establish a possible correlation between patient status, paraclinical examination and laboratory analyzes (approximation of carotid stenosis with the help of the two reports – establishing the prognosis of the ischemic stroke by calculating the two reports).

EXCLUSION CRITERIA:

- For both groups: any condition that can influence inflammatory markers:
  - infections,
  - febrile state,
  - solid neoplasms,
  - autoimmune diseases,
  - oncohematological diseases (lymphomas, multiple myeloma, etc.).

1.3 Results

1. Description of case and control batches

The mean age of the patients included in the study did not differ significantly, in the study group the mean age was 71 years, while in the control group the mean age was 67 years.
Three of the most frequent personal pathological antecedents encountered in the patients included in the study group were: arterial hypertension (73%), atrial fibrillation (38%) and type II diabetes (32%).

From the point of view of the correlations regarding the control group, we can conclude that there is a good, directly proportional, positive correlation with statistical significance (r=0.641, p<0.001) between the two ratios (NEU/LYM, PLR/ LYM) studied and between hsCRP and reports included in the study (Figure 1, 2, 3). The NIHSS score at admission correlates statistically significantly with the hsCRP collected in the first 24 hours after the onset of ischemic stroke symptoms (figure 4). At the same time, there is a weak-moderate correlation, directly proportional, between the degree of stenosis and the NEU/LYM ratio and a weak-moderate correlation, inversely proportional, between the degree of stenosis and the PLT/LYM ratio.

A statistically significant, directly proportional correlation with a Pearson correlation coefficient of 0.641 was observed between the two reports included in the study (Figure 1). At the same time, hs PCR correlates statistically significantly, directly proportionally, with a Pearson correlation coefficient of 0.424 with the NEU/LYM ratio (figure 2) and with the PLT/LYM ratio with a Pearson correlation coefficient of 0.468 (figure 3).

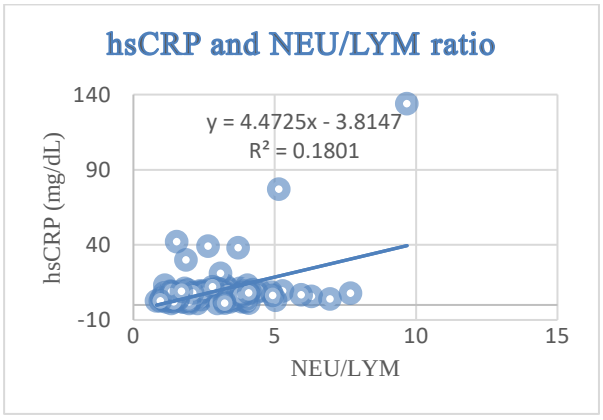


Figure 1 – Correlation between NEU/LYM ratio and PLT/LYM ratio

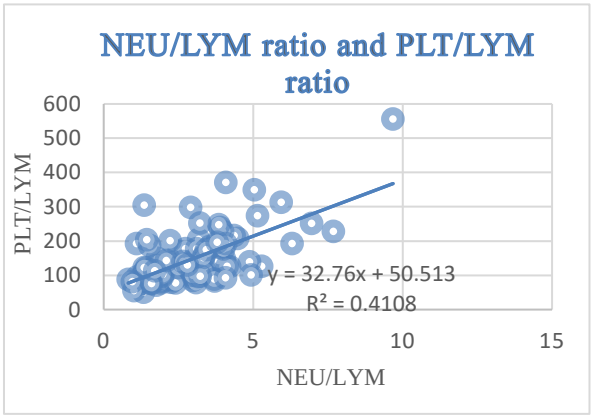


Figure 2 – Correlation between hsCRP and NEU/LYM ratio

One of the most important correlations of the present study is between hsCRP and the NIHSS score at admission (r = 0.296, p<0.001), directly proportional, positive correlation (figure 4).

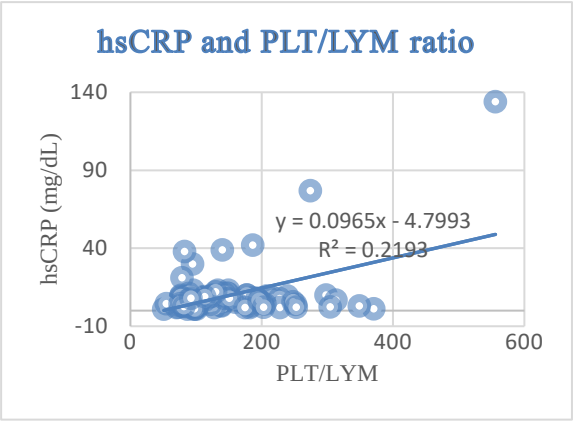


Figure 3 – Correlation between hsCRP and PLT/LYM ratio

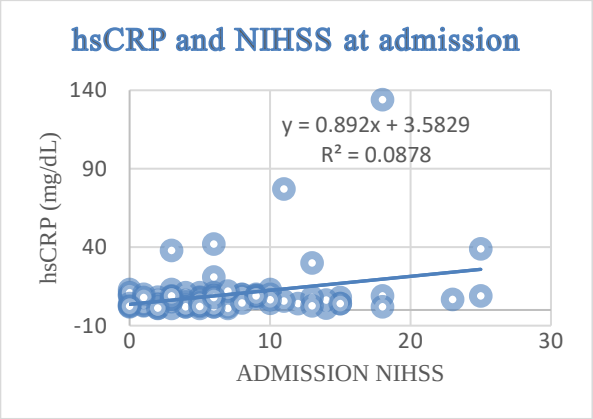


Figure 4 – Corelation between hsCRP and NIHSS at admission

The degree of carotid stenosis correlates moderately (tendency to a statistically significant value), directly proportionally, (r=0.188), with the NEU/LYM ratio and moderately, inversely proportionally with the PLT/LYM ratio, (r=-0.189), these ratios being able to be taken into account to monitor an already pre-existing stenosis or to consider the existence of carotid stenosis in patients who are not diagnosed but have these values changed for no other reason. At the same time, the NIHSS at discharge correlates statistically significantly with the degree of carotid stenosis, which shows us that disability is closely related to the quantification of carotid atheromatosis (figure 9).

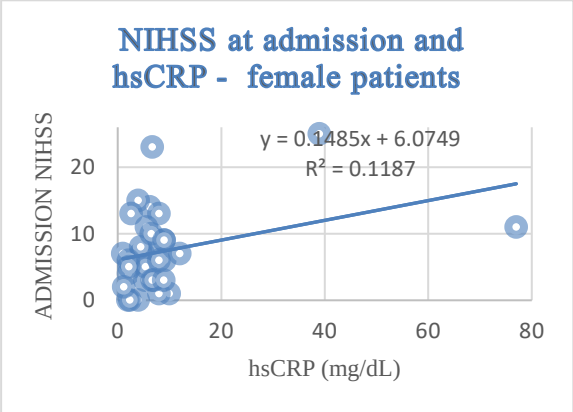


Figure 8 – Corelation between NIHSS at admission and hsCRP

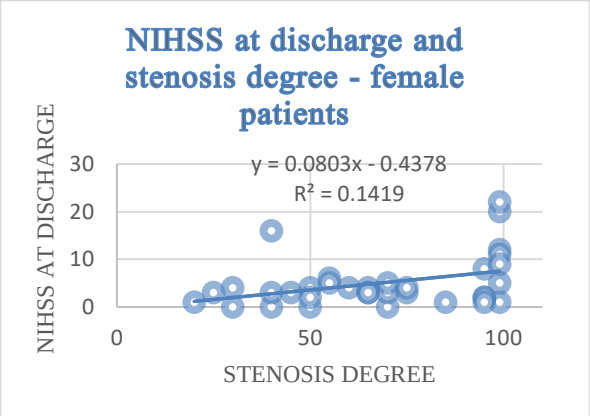


Figure 9 – Corelation between NIHSS at discharge and carotid stenosis degree

1.4 Conclusions

1. Female patients experienced ischemic strokes at older ages (76 years) compared to male patients (68 years). In the case of the other variables included in the study, no significant differences were observed between the two genders.

2. Female patients had more severe ischemic strokes with a mean NIHSS score of 7.34 compared to males who totaled a mean NIHSS score of 6.8 points.

3. The hsCRP variable correlates directly proportionally, statistically significantly, with the NIHSS at admission in patients in the study group, which is why we can conclude that it can be considered a reliable marker in terms of the evolution and prognosis of patients with ischemic stroke.

4. The ratios between neutrophils and lymphocytes (NEU/LYM) and platelets and lymphocytes (PLT/LYM) could be considered biomarkers for the prognosis and evolution of ischemic stroke, but further studies must be developed that take into account the infarcted vascular territory to be able to be correctly correlated with the score on the NIHSS scale.

5. Between the degree of carotid stenoses and the ratio of neutrophils to lymphocytes (NEU/LYM) there is a moderate, directly proportional correlation, respectively a moderate correlation, inversely proportional to the ratio of platelets to lymphocytes (PLT/LYM), which brings them into discussion as possible markers of carotid atheromatosis, but complementary studies are needed.

6. A neutrophil/lymphocyte ratio (NEU/LYM) value greater than or equal to 2.61 together with moderate (50-69%) or severe (≥70%) carotid stenosis may reflect a high risk of ischemic stroke .

7. The average value of the platelet/lymphocyte ratio (PLR) in the study group was significantly higher than in the control group (147.86, while in the control group 114.95), so we consider that this ratio can be taken into account consideration for dynamic evaluations of ischemic stroke severity.

8. Ischemic strokes in the anterior territory (middle cerebral artery, respectively anterior cerebral artery) - 72%, were more frequent in the study group, than those in the posterior territory - 28%.

9. A percentage of 59% of the patients included in the study were diagnosed with moderate-severe carotid stenoses (moderate stenoses quantified as a percentage of 50-69%, severe stenoses quantified as a percentage ≥ 70%). Most of these patients were male and smokers.

STUDY 2 – Evaluation of specific reactants (interleukin 6, interleukin 1β and TNF-alpha) of the acute phase in inflammation caused by acute ischemic stroke

2.1 Introduction

Working hypothesis

Contrary to recent therapeutic advances, ischemic stroke remains a neurological pathology with a huge socioeconomic impact worldwide [143]. Therapeutic approaches have primarily been directed to the preservation of neurons in the ischemic territory and currently available treatments, thrombolysis - intravenous recombinant tissue plasminogen activator and endovascular interventions - aim at rapid recanalization to restore oxygen and nutrient supply to the affected area [143] . In the core of the ischemic territory, clinically defined as the region with regional cerebral blood flow < 20%, acute neuronal death occurs rapidly within minutes to hours after occlusion due to an energy deficit resulting in intracellular ion imbalance, insufficiency mitochondrial. and activation of intracellular proteases, lipases, and ribonucleases, leading to rapid breakdown of cellular structural elements and loss of cellular integrity. However, outside the ischemic core, brain tissue is still partially perfused, albeit at a reduced rate. This region, referred to as the ischemic penumbra, is often defined by a reduced perfusion rate, which is, however, greater than that observed in the ischemic core [144]. However, even if blood flow is restored, penumbra neurons face major challenges to their survival, such as excitotoxicity and inflammation. Inflammation, initiated by stagnant blood flow, activation of intravascular leukocytes, and release of proinflammatory mediators from ischemic endothelium and brain parenchyma, has the potential to increase tissue damage. While many aspects of inflammation are beneficial and aimed at restoring tissue homeostasis, collateral damage caused by the acute inflammatory response contributes to ischemic damage.

Purpose of the study:

Inflammation in ischemic stroke is an intensely debated topic in studies conducted in recent years, which can lead to worsening of the patient's status and can be a target for future therapies regarding this pathology. The aim of the present study is to determine whether there is a correlation between biomarkers of inflammation and the severity of ischemic stroke, as well as the prognosis of patients who have suffered cerebral ischemia.

The specific objectives of the study:

The main objective – evaluation of the correlation between serum inflammatory markers – interleukin 6, interleukin 1β and TNF alpha (specific acute phase reactants) and the severity of ischemic stroke, as well as the prognosis of patients with this pathology.

Secondary Objectives:

- Studying a possible correlation between carotid atherosclerosis and these markers.
- Establishing the risk factors and comorbidities of patients who have already suffered an ischemic stroke, highlighting the main comorbidities that could contribute to a new recurrence.
- Compilation of a risk score of the prognosis of patients who have suffered an ischemic stroke, as well as the risk of a recurrence of the stroke with real applicability, following the corroboration of the accumulated data.

2.2 Material and methods

Choosing the study group:

The study was conducted between January 1 and December 31, 2021 at the Sibiu County Emergency Clinical Hospital, respectively the Department of Neurology. The study was approved by the ethics committee of the Sibiu County Emergency Clinical Hospital and the Lucian Blaga University of Sibiu (attached to this paper). Each patient included in the study signed an informed consent (attached to this paper).

Stages of the study:

The research groups included adult subjects who suffered an acute ischemic stroke (onset < 24 hours prior to presentation in the Sibiu Emergency Department) and who were consecutively admitted to the Sibiu County Emergency Clinical Hospital. The stages of the study were clearly explained to all patients included in the study, prior to signing the informed consent:

- neurological assessment performed by a neurologist at the time and during hospitalization (neurological examination + NIHSS score + Rankin score);
- performing specific blood tests (inflammatory markers) in addition to the standard tests performed during hospitalization;

The patients were divided into two groups:

• A RESEARCH LOT divided into two sublots:

• patients with ischemic stroke and SARS-COV2 infection (group compared with a subgroup of patients with ischemic stroke, without associated SARS-COV2 infection)

• patients with ischemic stroke and SARS-COV2 negative

• A CONTROL GROUP: group of patients with no personal pathological history of ischemic stroke, of age and gender comparable to the research group (to prove that the levels of the collected analyzes are within physiological limits).

Data collected retrospectively and at the time of inclusion in the study: age, gender, personal pathological history (associated conditions), paraclinical data (cranial CT), PCR test.

Data collected during the research: biological: IL-6, TNF alpha, IL-1 β and paraclinical: extracranial carotid Doppler ultrasound.

Inclusion criteria in the study:

- Adult patients, who were admitted to the Sibiu County Emergency Clinical Hospital, with suspicion of ischemic stroke

- Neurological symptoms and signs that raise the suspicion of an ischemic stroke at the onset (onset < 24 hours)

- Brain imaging to rule out a brain tumor or hemorrhagic stroke

Exclusion criteria from the study:

- Any medical pathology that can influence markers of inflammation: infections, autoimmune diseases, neoplasias, hematological diseases (lymphoma, multiple myeloma, etc.), rheumatological diseases (rheumatoid arthritis).

- Patients who have recently (last 30 days) undergone treatment with corticosteroids or immunosuppressants.

- Patients known in the last 180 days with acute myocardial infarction, myocarditis or acute ischemic stroke

- Patients who suffered trauma before presenting to the on-call service and who were noted at the time of admission.

Collection of biological samples and measurement of biomarkers

All samples were collected in EDTA vacutainers, centrifuged at 1500 ×g for 15 minutes and subsequently frozen at −80°C. The biomarkers IL-6, IL-1 β and TNF alpha were processed from the samples. Samples that did not conform were excluded.

As imaging markers, extracranial doppler ultrasound of the carotid arteries was performed, through which the degree of carotid atheromatosis was established:

a. early carotid atheromatosis – small atheromatous plaques arranged at the level of the ICA

b. moderate carotid atheromatosis – carotid stenosis (ICA) of 50-69%

c. severe carotid atheromatosis – carotid stenoses (ICA) >70%

2.3 Results

In the case of the study group, the most common risk factor was arterial hypertension, which was found in 62.5% of patients, followed by atrial fibrillation (32.14%) and type II diabetes (19.64%) (table 10).

Inflammatory markers, namely IL-6, TNF-alpha and IL1β (first collection), correlated positively, directly proportionally, with NIHSS at admission. Their values were significantly increased in patients with extensive ischemic strokes, who scored high on the NIHSS score (figure 15-19).

The IL6 biomarker – collection on day 1 (figure no. 15) and collection on day 7 (figure no. 16) correlates statistically significantly, directly proportionally, with r = 0.951, respectively r = 0.940, p < 0.001, with the NIHSS score at admission, respectively the severity of the ischemic stroke.

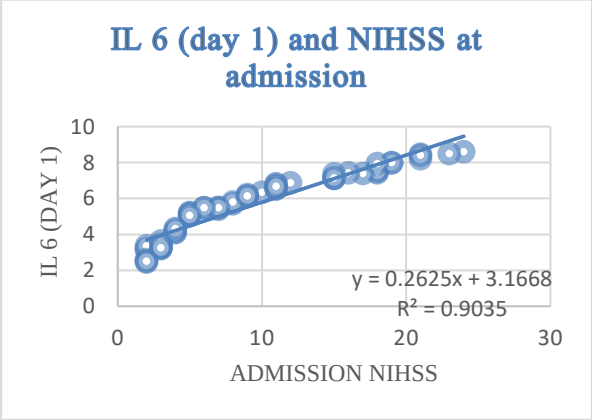


Figure 15 – Corelation between IL-6 (day 1) and NIHSS at admission

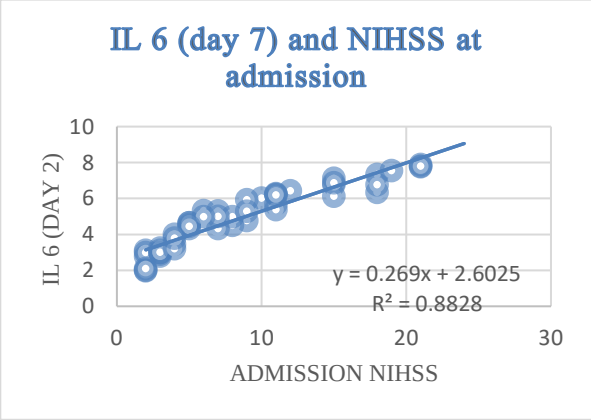


Figure 16 – Corelation between IL-6 (day 7) and NIHSS at admission

The IL1β biomarker, collected on day 1, correlates directly proportionally, statistically significantly, r = 0.424, p < 0.001, with the NIHSS score at admission, in patients with acute ischemic stroke (figure 17).

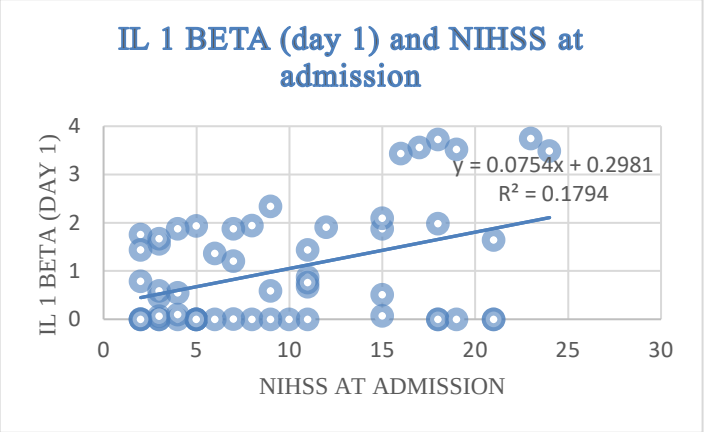


Figure 17 – Corelation between IL-1 β (day 1) and NIHSS at admission

Similarly, the biomarker TNF-alpha, both collections, day 1 and day 7, respectively, correlates statistically significantly, directly proportionally, r = 0.972 and r = 0.671, respectively, p < 0.001, with the total score on the NIHSS scale by patients who underwent an acute ischemic stroke (figure 18 and figure 19).

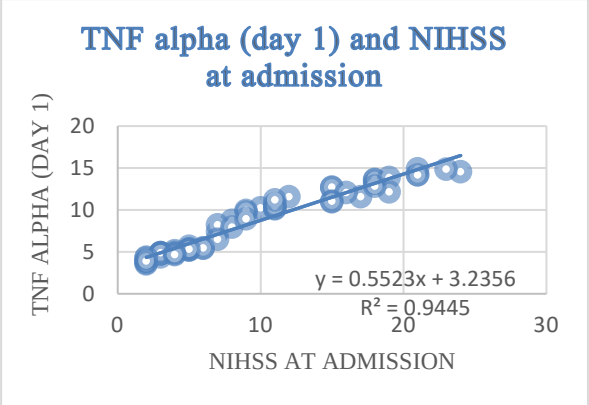


Figure 18 – Corelation between TNF alpha (day 1) and NIHSS at admission

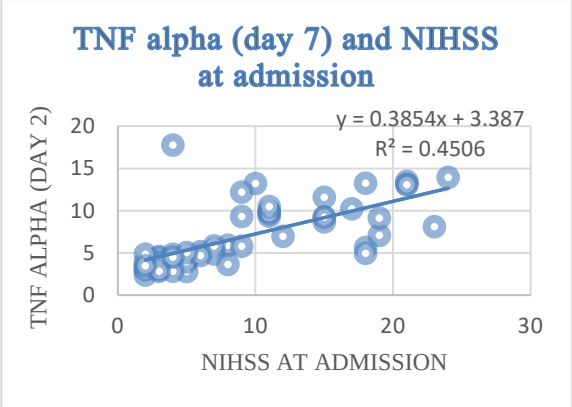


Figure 19 – Corelation between TNF alpha (day 7) and NIHSS at admission

Regarding the NIHSS at discharge, inflammatory markers were positively correlated, respectively directly proportional to the score obtained on this scale by patients who suffered ischemic strokes. IL6 and TNF alpha, both collections, were positively correlated with NIHSS at discharge (figure 21-24), (the values of these markers increased in patients who worsened and obtained a higher NIHSS at discharge than in day 1, respectively decreased in patients who improved and obtained a lower score compared to day 1, respectively the day of admission). Among the markers considered, the second collection of TNF-alpha (ie the collection on day 7) had the best correlation with the NIHSS at discharge in the patients included in the study.

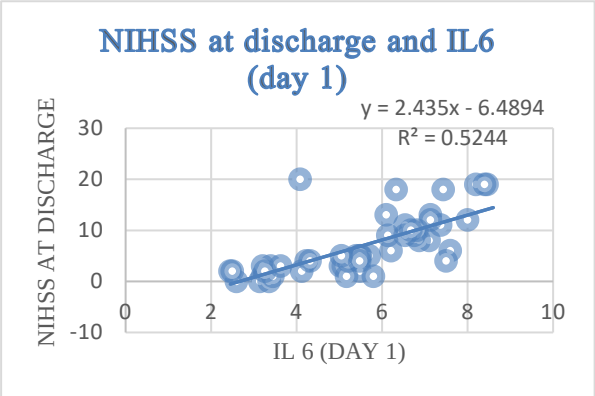


Figure 21 – Corelation between IL-6 (day 1) and NIHSS at discharge

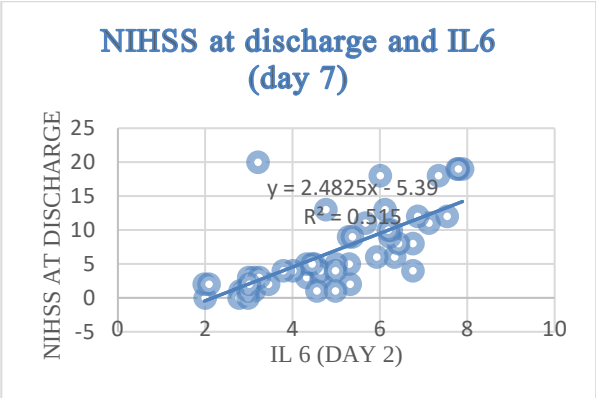


Figure 22 – Corelation between IL-6 (day 7) and NIHSS at discharge

Both collections of the TNF-alpha biomarker correlate statistically significantly, directly proportionally, r = 0.746, respectively r = 0.968, p < 0.001, with the NIHSS score at discharge (figure 23). The second collection of TNF-alpha has the best correlation with this score, which is practically the most reliable biomarker regarding the prognosis of ischemic stroke patients 7 days after the onset (figure 24).

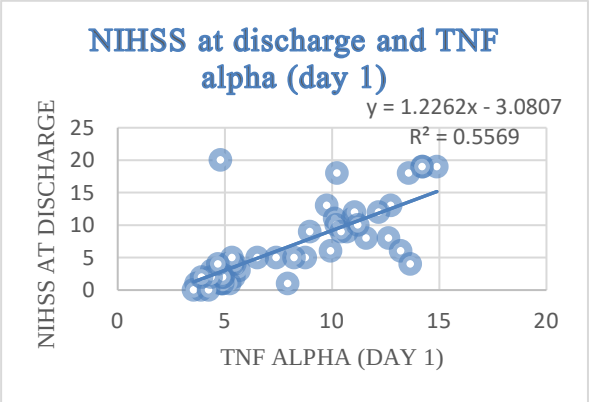


Figura 23 – Corelation between TNF-alpha (day 1) and NIHSS at admission

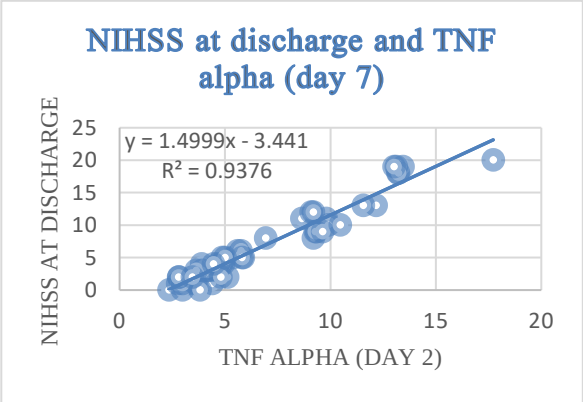


Figura 24 – Corelation between TNF-alpha (day 7) and NIHSS at admission

However, there are significant differences between the score obtained on the Rankin scale at discharge and the type of ischemic stroke (p=0.001). Patients who had anterior territory ischemic strokes had a higher total Rankin scale score compared to those who had posterior territory ischemic strokes or lacunar strokes (Figure 34).

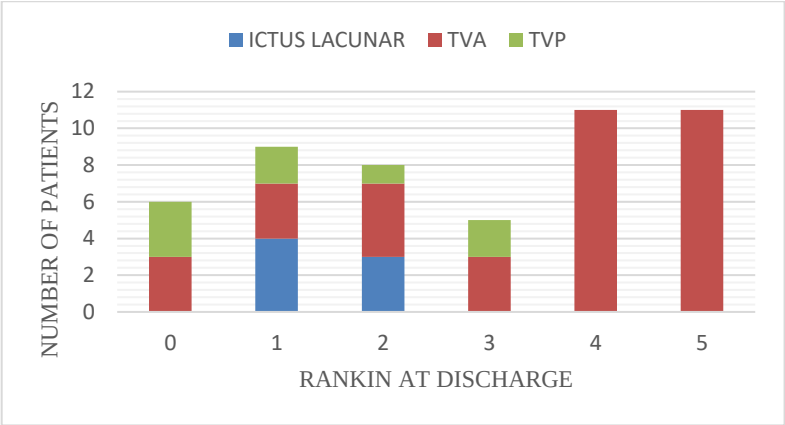


Figure 34 – Rankin at discharge according to stroke type

Correlation of IL1beta and extracranial carotid artery Doppler ultrasound

(*=statistically significant (p<0.05), **=highly statistically significant (p<0.001)

VARIABLE	NIHSS at admission	CAROTID ULTRASOUND
All patients		
IL 1 BETA (day 1)	0,238	,529**
IL 1 BETA (day 7)	0,102	,653**

Table 22 – Corelation between IL1β (day 1 and day 7) and NIHSS at admission, IL1β and carotid ultrasound

The inflammatory marker IL1β, respectively both collections, correlated positively, directly proportionally, statistically significantly with the degree of carotid atheromatosis ($p < 0.001$).

Severe stenoses of the internal carotid artery, determined by means of extracranial doppler ultrasound, correlated statistically significantly, directly proportionally, with the IL1β biomarker value, both collections.

CORRELATIONS OF BIOMARKERS OF INFLAMMATION IN PATIENTS WITH SARS-COV 2 INFECTION

The patients included in the study were divided into two groups, namely patients with SARS-COV 2 infection and patients without SARS-COV2 infection. The age of the patients included in both groups was on average 70 years, with an NIHSS at admission averaging 12.5 points in patients with SARS-COV 2 infection and 11 points in those without SARS-COV 2 infection.

In the group of patients without SARS-COV2 infection, 32.5% of patients who suffered an ischemic stroke received thrombolytic treatment, compared with 15% in the group with SARS-COV2 infection. SARS-COV2 infection was significantly correlated with the death of patients with ischemic stroke included in the study, OR=4.2 (95%CI 1.35 - 13.065), the risk of a patient with SARS COV 2 infection dying being 4.2 times higher than those without associated SARS-COV2 infection.

The NIHSS at admission correlates significantly with the severity score described on the computed tomography of the chest in patients with ischemic stroke and SARS-COV 2 infection: $r=0.742$ ($p<0.001$) – very good correlation, statistically significant, directly proportional (when the NIHSS score increases, the chest CT severity score also increases).

An NIHSS score >15 significantly influences death, those who total a score above 15 on the NIHSS scale have a 9.16 times greater risk of dying. It correlates in the multivariate analysis with fibrinogen and IL 6, i.e. all 3 variables influence death at the same time.

In the case of patients with SARS-COV 2 infection, the variables fibrinogen, ESH, D-dimers and IL6 were positively correlated with the degree of severity of ischemic stroke, positive correlation, directly proportional.

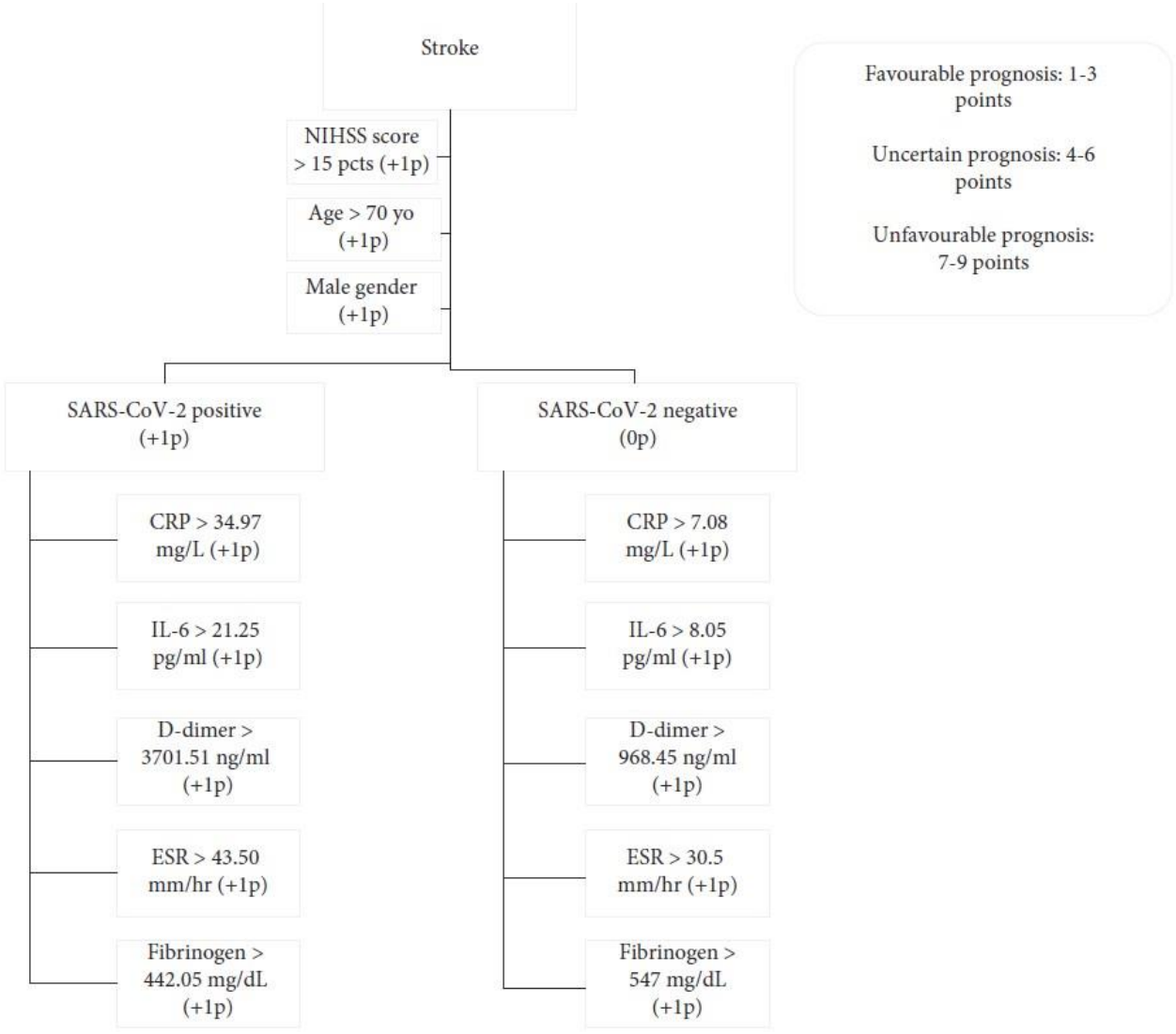


FIGURE 6: Severity and outcome prediction score.

1.5 Conclusions

1. In patients with acute ischemic stroke inflammation markers, respectively IL6 (first collection - day 1 and second collection - day 7), TNF-alpha (first collection - day 1 and second collection - day 7), IL1β (first collection – day 1) is positively correlated, directly proportional to the score totaled by patients with acute ischemic stroke on the NIHSS scale at admission.

2. There is no significant correlation, in patients with acute ischemic stroke, between NIHSS on admission and IL1β at the second collection - day 7.

3. In patients with acute ischemic stroke, the NIHSS at discharge correlates positively, directly proportionally with inflammation markers, respectively IL6 (first collection - day 1 and second collection - day 7), TNF-alpha (first collection - day 1 and second harvest – day 7). Discharge NIHSS does not correlate with IL1β.

4. IL6 and TNF-alpha are the markers that had the best correlations with both NIHSS at admission and NIHSS at discharge in patients with acute ischemic stroke.

5. IL1β (first collection – day 1 and second collection – day 7) correlates positively and directly proportionally with moderate (50-69%) and severe carotid stenoses (≥ 70%) – (cut off value – 1.0375)

6. RANKIN scale at discharge correlates positively, directly proportionally, with IL6 and TNF-alpha. TNF-alpha the second collection having the best correlation with this scale among all the markers included in the study, thus concluding that it can be considered a faithful marker of the disability prognosis of patients with ischemic stroke.

7. Patients with ischemic stroke in the anterior vascular territory have higher values of inflammation markers compared to patients with ischemic stroke in the posterior territory, respectively compared to those who suffered a lacunar stroke.

8. At the onset of acute ischemic stroke (the first 24 hours from the onset of symptoms), cut off values of 5.310 (IL6), 1.938 (IL1β) and 6.095 (TNF-alpha) differentiate between the territories affected by cerebral ischemia, respectively the previous vascular territory and posterior vascular territory.

9. Patients with acute ischemic stroke in the anterior vascular territory total a higher score on the Rankin scale at discharge, having a more pronounced disability compared to patients with acute ischemic stroke in the posterior territory or lacunar stroke.

10. IL6, TNF-alpha and IL1β biomarker values in the first 24 hours can be considered predictive of patient death (biomarker levels collected in the first 24 hours were significantly higher in patients who died).

11. SARS-COV 2 infection and NIHSS ≥ 15 in ischemic stroke patients significantly influence death. The death of these patients also correlates with IL6 and fibrinogen.

12. Patients with acute ischemic stroke and SARS-COV 2 infection have a 4.2 times higher risk of death than patients with acute ischemic stroke without associated SARS-COV 2 infection.

13. Patients with acute ischemic stroke, SARS-COV2 infection, and NIHSS ≥ 15 have a 9.16 times higher risk of dying compared to patients with acute ischemic stroke without associated SARS-COV2 infection.

14. NIHSS at admission in patients with acute ischemic stroke and SARS-COV2 infection correlates significantly, directly proportionally, with chest CT severity score.

15. The severity of acute ischemic stroke in patients with SARS-COV2 infection correlates significantly, positively, directly proportionally with IL6, ESH, fibrinogen and D-Dimers.

STUDY 3 – Importance of thyroid dysfunction in acute ischemic stroke

3.1 Introduction

Thyroid dysfunction is a relatively common pathology, but until now the long-term effects on morbidity and mortality are not fully elucidated.

Thyroid-stimulating hormone (TSH) regulates the synthesis and release of thyroid hormones through the negative feedback loop of the hypothalamic-pituitary-thyroid axis. Thus, TSH is an important marker of thyroid dysfunction: elevated TSH indicates hypothyroidism and TSH below physiological limits indicates hyperthyroidism [195].

Subclinical thyroid dysfunction is defined by serum thyroid-stimulating hormone (TSH) values outside the reference range, but with normal free thyroxine (T4) concentrations, as well as free triiodothyronine (T3) in case of subclinical hyperthyroidism [196].

Thyroid dysfunction in the subclinical range is very common, with a prevalence of subclinical hypothyroidism ranging from 4 to 14% in adults, this percentage increasing with age. Subclinical hyperthyroidism is uncommon in the general population with a prevalence ranging from 0.7% [16] to 10% in women [197].

In hyperthyroidism, the increase in thyroid hormones leads to an increase in cardiac contractility, blood flow and heart rate and reduces vascular resistance. This can lead to cardiac hypertrophy and arrhythmia. The effect of subclinical thyroid dysfunction on cardiovascular disease risk factors may be less pronounced [196]. Risk factors can, however, increase the risk of ischemic heart disease, stroke, and mortality. One certainty, however, is the fact that the clinical importance, especially in subclinical thyroid dysfunction, is still a big question mark in current research.

3.2 Material and methods

The study included 80 adult patients who were diagnosed with latent hyperthyroidism (low TSH, FT4 within reference limits) and latent hypothyroidism (increased TSH, FT4 within reference limits), consecutively admitted to the Emergency County Clinical Hospital Sibiu, Neurology clinic, with a diagnosis of acute ischemic stroke (onset symptomatology. The study was carried out over 3 years (January 2020 – December 2022), the recruitment period was 24 months, the remaining 12 months being allocated to follow-up patients.

Patients were divided into two study subgroups:

• A research group – which included patients with thyroid pathology subdivided into:

♣ Sublot 1 – patients with subclinical hyperthyroidism – FT4 within reference limits, low TSH.

♣ Sublot 2 – patients with subclinical hypothyroidism – FT4 within reference limits, elevated TSH.

Data collected retrospectively and at the time of inclusion in the study: age, gender, APP (associated conditions), paraclinical data (extracranial carotid Doppler ultrasound), biological: blood count, serum levels of FT4, TSH, cholesterolemia.

Data collected during research: correlations between hypo- and hyperthyroidism-specific biological markers and cerebral ischemia.

As imaging markers, extracranial doppler ultrasound of the carotid arteries was performed, through which the degree of carotid atheromatosis was established:

a. early carotid atheromatosis – small atheromatous plaques arranged at the level of the ICA (echo 0)

b. moderate carotid atheromatosis – carotid stenosis (ICA) of 50-69% (echo 1)

c. severe carotid atheromatosis – carotid stenoses (ICA) >70% (echo 2)

Inclusion criteria:

• Patients without chronic ischemic stroke in the personal pathological history and without known thyroid pathology at the time of inclusion in the study

• Patients with acute ischemic stroke, with onset of symptoms ≤ 24 hours.

• Subclinical hyperthyroidism: TSH level – 0.01 – 0.04 mU/L

• Subclinical hypothyroidism: TSH level ≤ 10 mU/L

Exclusion criteria:

• Patients who were on statin treatment

• Known thyroid and endocrinological pathology

• Chronic ischemic stroke

3.3 Results

Most of the patients included in the study were female (83.75%). The most common thyroid pathology was subclinical hypothyroidism, 67 patients were diagnosed de novo with subclinical hypothyroidism at the time of admission, and 13 patients with subclinical hyperthyroidism. From the point of view of neurological pathology, 45% of the patients included in the study suffered an ischemic stroke in the anterior vascular territory and only 11% lacunar strokes.

There is a statistically significant association ($p < 0.05$) between cholesterol and TSH (table 36). There are no significant associations between TSH and the other variables included in the study, namely the type of ischemic stroke, the gender of the patients or the degree of carotid atheromatosis.

A percentage of 91% of the patients included in the study had increased cholesterol levels, correlated with high TSH levels (figure 62).

Given the fact that a greater number of patients suffered ischemic strokes in the anterior vascular territory, this was also found in the diagnosis of subclinical hypothyroidism, respectively, most patients with subclinical hypothyroidism were diagnosed with cerebral ischemia in the anterior territory (figure 63).

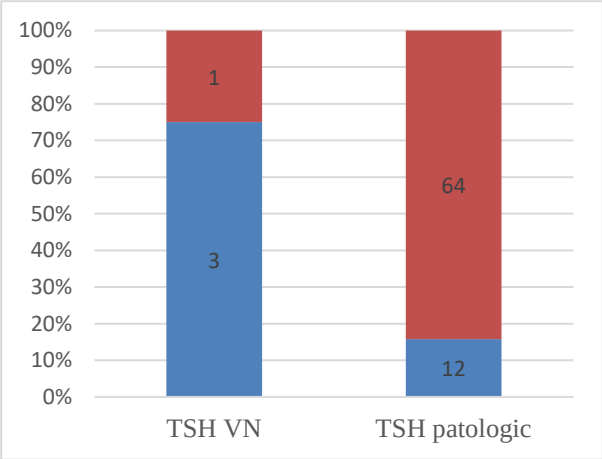


Figure 62 – Corelation between TSH and cholesterol

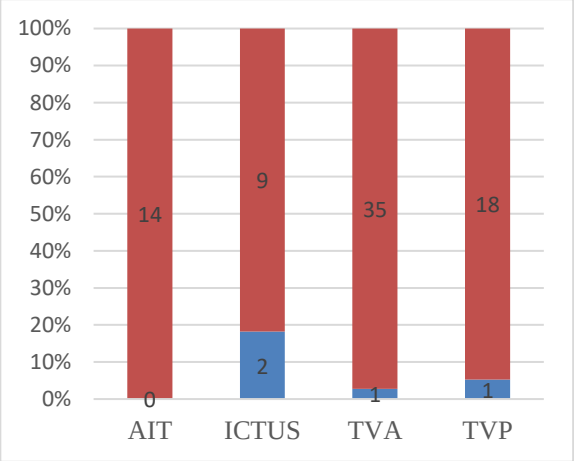


Figure 63 – Corelation between TSH and stroke type

All patients included in the study underwent extracranial carotid artery Doppler ultrasound at the time of admission and a statistically significant, directly proportional correlation was observed between TSH levels and the degree of carotid stenosis (figure 64).

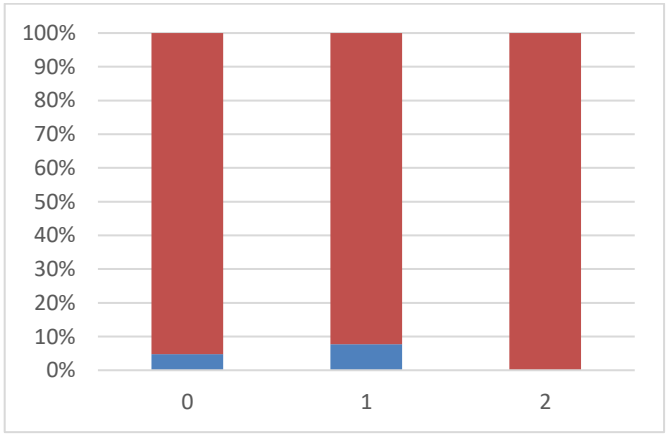


Figure 64 – Correlation between serum TSH levels and degree of internal carotid artery stenosis

Between TSH and total cholesterol there is a very good positive linear correlation, statistically significant ($r=0.794$, $p<0.001$) (figure 65).

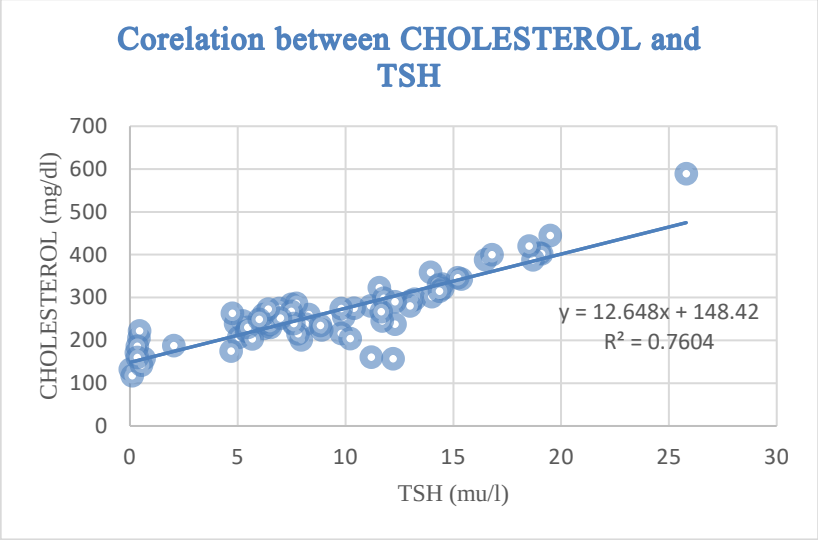


Figure 65 – Significant correlation between serum TSH levels and total cholesterol levels

TSH correlated significantly, directly proportionally, with NIHSS at admission in patients with ischemic stroke in the anterior territory, but no significant correlation was observed between it and TIA, lacunar stroke, or stroke in the posterior territory (Figure 66).

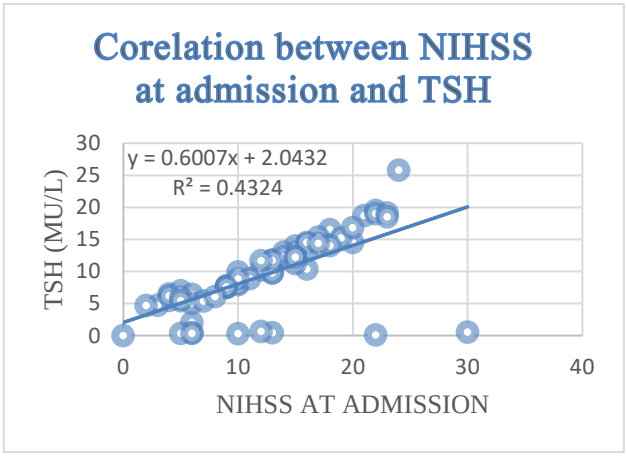


Figura 66 – Corelation between NIHSS at admission and TSH levels

3.4 Conclusions

1. Female patients are the majority in terms of the diagnosis of subclinical hypothyroidism, 83.75% of the patients included in our study being diagnosed with this pathology.
2. A percentage of 45% of the patients included in the study suffered an acute ischemic stroke in the previous vascular territory.
3. In the case of patients with acute ischemic stroke in the anterior vascular territory, serum TSH levels correlate significantly, directly proportionally, with the severity of cerebral ischemia, respectively with the NIHSS score at admission.
4. Serum TSH levels at admission correlated statistically significantly, directly proportionally, with the RANKIN scale at discharge in patients with acute ischemic stroke in the anterior vascular territory, thus constituting a predictive factor for the degree of disability in these patients.

5. TSH levels correlated significantly, directly proportionally, with the degree of carotid stenoses. A TSH value ≥ 4.7 mIU/L differentiates between patients without carotid atheromatosis and those with moderate-severe carotid atheromatosis.

6. Subclinical hypothyroid patients with high TSH levels were diagnosed with hypercholesterolemia (significant, very good, directly proportional correlation between TSH and total cholesterol).

7. The majority of patients diagnosed with subclinical hyperthyroidism (78%) were diagnosed with de novo atrial fibrillation at the time of admission.

8. In patients with subclinical hyperthyroidism, TSH did not correlate statistically significantly with NIHSS at admission.

9. Subclinical hyperthyroidism is a protective factor for echo 1 (carotid stenosis 50-69%) or echo 2 (carotid stenosis ≥ 70%): OR=0.211 (95%CI 0.043-0.996), p=0.038, meaning that patients with subclinical hyperthyroidism are less often diagnosed with moderate or severe carotid atheromatosis.

10. During the course of the study, 9 recurrences (13.43%) of ischemic stroke were observed in patients with subclinical hypothyroidism and 1 patient (7.69%) with subclinical hyperthyroidism presented a TIA CS (patient diagnosed with atrial fibrillation, but with correct medical treatment followed at home). We therefore consider drug treatment necessary also in patients with subclinical thyroid pathology.

GENERAL CONCLUSIONS

1. Most frequently, ischemic strokes affect the anterior territory, female patients presenting ischemic strokes at older ages and with increased severity, with higher NIHSS score compared to male patients.

2. Three of the most common personal pathological antecedents in patients with acute ischemic stroke are hypertension, atrial fibrillation and type II diabetes.

3. The hsCRP biomarker correlates positively, directly proportionally, statistically significantly, with NIHSS of patients at admission, which makes it a reliable marker regarding the evolution and prognosis of patients with ischemic stroke.

4. Neutrophil-to-lymphocyte and platelet-to-lymphocyte ratios are influenced by cerebral ischemia and correlate positively, directly proportional to the severity of ischemic stroke, but further studies need to be developed to precisely establish the details of this correlation.

5. Patients with moderate-severe stenosis (≥50%) of the internal carotid artery associated with a neutrophil to lymphocyte ratio (NEU/LYM) greater than 2.61 are at high risk of stroke.

6. Markers of inflammation, namely interleukin 6 (IL6), interleukin 1β (IL1β), tumor necrosis factor alpha (TNF-alpha) collected in the first 24 hours after the onset of ischemic stroke symptoms, correlate statistically significantly, directly proportionally with the severity of cerebral ischemia and the degree of disability of these patients.

7. Interleukin 6 (IL 6) and tumor necrosis factor alpha (TNF-alpha) correlate statistically significantly, directly proportional to the NIHSS score at discharge, which means that these markers of inflammation can be used dynamically to establish the evolution and the prognosis of patients with ischemic stroke both during hospitalization and at discharge.

8. Tumor necrosis factor (TNF-alpha) is the most faithful marker in terms of the degree of disability at discharge of patients with ischemic stroke, having a very good correlation, directly proportional, with the RANKIN scale at discharge.

9. The degree of internal carotid artery stenoses correlates significantly, directly proportionally, with the inflammation marker IL1β; the cut off value – 1.0375 can differentiate between moderate carotid stenoses (50-69%) and severe carotid stenoses (≥70%).

10. Ischemic strokes in the anterior vascular territory produce higher inflammation marker values in the first 24 hours after the onset of symptoms compared to cerebral ischemias in the posterior territory, which is why the cut-off values of the markers included in the study (IL6 – 5,310 pg /mL, IL1β –

1.938 pg/mL and TNF-alpha – 6.095 pg/mL) can make the difference, right from admission, between the affected territories.

11. The high inflammation is maintained even 7 days after the onset of the stroke, thus being able to make the differential diagnosis with certainty between the affected territory and even stroke-mimics (cut off values – IL6 – 4.705 pg/mL, IL1β – 1.906 pg /mL and TNF-alpha – 4,210 pg/mL). At the same time, the values of the inflammatory markers decrease progressively from the onset of the stroke.

12. The very high levels of inflammation biomarkers (cut off values – IL6 – 8.24 pg/mL, IL1β – 3.54 pg/mL and TNF-alpha – 14.22 pg/mL) and an NIHSS ≥ 21 points in the first 24 hours from the onset of stroke symptoms can be considered predictive of patients' death.

13. SARS-COV 2 infection and NIHSS ≥ 15 in patients with ischemic stroke significantly influence death, with a 9.6 times higher risk of death compared to patients with acute ischemic stroke without SARS infection -COV 2 associated.

14. If NIHSS score ≤ 15 points, patients with acute ischemic stroke and SARS-COV 2 infection have a 4.2 times higher risk of death than patients with acute ischemic stroke without associated SARS-COV 2 infection.

15. NIHSS at admission in patients with acute ischemic stroke and SARS-COV2 infection correlates significantly, directly proportionally, with the severity score of chest CT performed at admission. Practically, lung damage in SARS-COV 2 infection is a negative factor in terms of cerebral ischemia.

16. The severity of acute ischemic stroke in patients with SARS-COV2 infection correlates significantly, positively, directly proportionally with IL6 (cut-off value – 21.25 pg/mL), VSH (cut-off value – 43.50 mm/h) , fibrinogen (cut-off value – 442.05 mg/dL) and D-Dimers (cut-off value – 3701.51 ng/mL).

17. In the case of patients with acute ischemic stroke, without associated SARS-COV 2 infection, the severity of cerebral ischemia correlates significantly, directly proportionally, with hsPCR (7.08 mg/L), fibrinogen (547 mg/dL), ESR (30.5 mm/h), D-dimers (968.45 ng/mL), IL-6 (8.05 pg/mL).

18. Thyroid pathology, namely subclinical hypothyroidism, can influence the severity of stroke, which is why thyroid hormone replacement therapy should be considered in these patients.

19. The serum levels of thyroid stimulating hormone (TSH – collected in the first 24 hours after the onset of cerebral ischemia symptoms) correlate statistically significantly, directly proportionally, with

the NIHSS score at admission of patients with acute ischemic stroke in the anterior vascular territory and subclinical hypothyroidism.

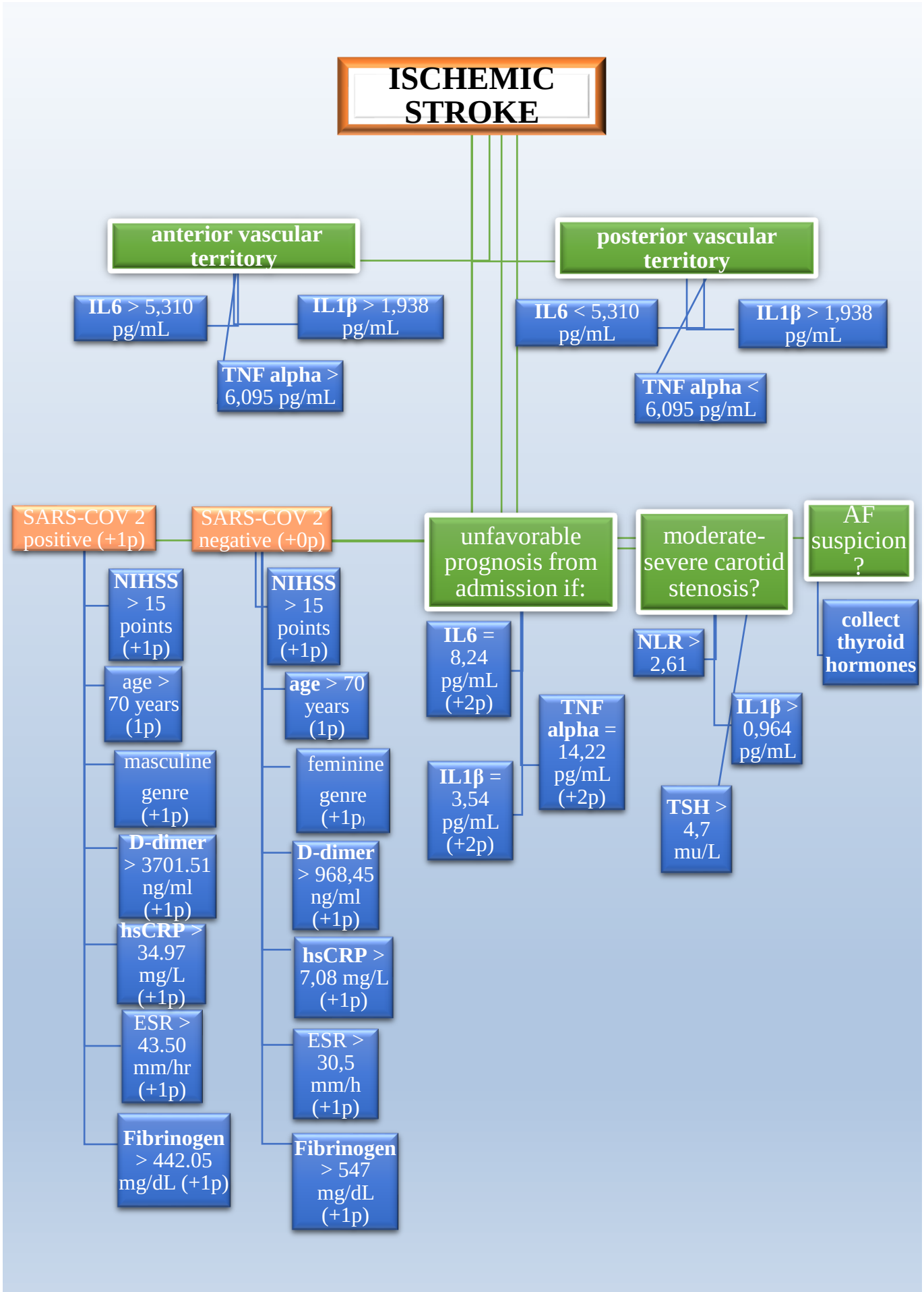
20. Thyroid stimulating hormone (TSH) correlates statistically significantly, directly proportionally, with total cholesterol levels in 82% of the patients included in the study.

21. Thyroid stimulating hormone values can be predictive for the degree of disability of patients with acute ischemic stroke, these values correlating directly proportionally, statistically significantly with the RANKIN scale at discharge.

22. The degree of internal carotid artery stenoses correlates significantly, directly proportionally, with thyroid stimulating hormone (TSH), with serum levels $\geq$ 4.7 mIU/L being found in moderate and severe carotid stenoses.

23. Subclinical hyperthyroidism can be considered a protective factor for moderate-severe carotid stenoses.

24. During the course of the study, 9 recurrences (13.43%) of ischemic stroke were observed in patients with subclinical hypothyroidism and 1 patient (7.69%) with subclinical hyperthyroidism presented a TIA CS (patient diagnosed with atrial fibrillation, but with correct medical treatment followed at home). We therefore consider it necessary to institute drug treatment in patients with subclinical thyroid pathology.


ISCHEMIC STROKE
anterior vascular territory
posterior vascular territory
IL6 > 5,310 pg/mL
IL1β > 1,938 pg/mL
TNF alpha > 6,095 pg/mL
IL6 < 5,310 pg/mL
IL1β > 1,938 pg/mL
TNF alpha < 6,095 pg/mL
SARS-COV 2 positive (+1p)
SARS-COV 2 negative (+0p)
unfavorable prognosis from admission if:
moderate-severe carotid stenosis?
AF suspicion ?
collect thyroid hormones
NIHSS > 15 points (+1p)
age > 70 years (1p)
masculine genre (+1p)
D-dimer > 3701.51 ng/ml (+1p)
hsCRP > 34.97 mg/L (+1p)
ESR > 43.50 mm/hr (+1p)
Fibrinogen > 442.05 mg/dL (+1p)
NIHSS > 15 points (+1p)
age > 70 years (1p)
feminine genre (+1p)
D-dimer > 968,45 ng/ml (+1p)
hsCRP > 7,08 mg/L (+1p)
ESR > 30,5 mm/h (+1p)
Fibrinogen > 547 mg/dL (+1p)
IL6 = 8,24 pg/mL (+2p)
IL1β = 3,54 pg/mL (+2p)
TNF alpha = 14,22 pg/mL (+2p)
NLR > 2,61
IL1β > 0,964 pg/mL
TSH > 4,7 mu/L

Personal contributions and elements of originality of the thesis

The studies included in this thesis aim to help clinicians with a set of biomarkers that, together with other risk factors, compose a severity and prognostic score in patients who have suffered an acute ischemic stroke. It is the first study in the country to address this topic and which, at the same time, tries to correlate biological biomarkers with the severity of carotid atherosclerosis.

We aimed to compile this score from accessible biomarkers that can be harvested dynamically for better accuracy of the final result.

From a socio-economic point of view, although the cost of these analyzes may seem high, some of the biomarkers included in the study are collected as routine analyzes in patients with acute ischemic stroke. However, their application can be of great help in preventing complications, relapses or even de novo cerebral ischemia.

We believe that the present work is a starting point regarding biological biomarkers of acute ischemic stroke that complement and not exclude biomarkers from the imaging sphere.

Limits of own research

First, one of the important limitations of the present study is the relatively small number of patients enrolled for the proposed research target.

The biomarkers were collected 24 hours after the onset of symptoms and 7 days later, without continuing to follow up the patients dynamically (at 3 months, at 6 months, at one year).
In future research, personal pathological antecedents should also be taken into account, such as hypertension or diabetes, which according to the latest publications in the specialized literature can influence markers of inflammation. At the same time, the body mass index of the patients included in the study should be taken into account and corroborated with the results of the markers, given that recent studies claim that obese patients may have increased serum levels of interleukins.

Ischemic stroke remains one of the challenges faced by neurologists, both in terms of treatment and in terms of the uncertainty of the patient's prognosis.

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