UNIVERSITATEA LUCIAN BLAGA DIN SIBIU

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ABSTRACT OF DOCTORAL THESIS

CORRELATIONS BETWEEN NONINVASIVE EXPLORATION OF NONALCOHOLIC FATTY LIVER DISEASE, COMPONENTS OF THE METABOLIC SYNDROME AND SYSTEMIC ATHEROSCLEROSIS

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CONTENTS

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects a quarter of the world's population (1)and could become the leading cause of liver transplantation by 2030.(2) Metabolic syndrome (MetS), characterised by abdominal obesity, dyslipidemia, hyperglycemia and hypertension(3)is on the rise globally, along with obesity and diabetes.(4) The presence of metabolic syndrome and NAFLD indicates increased cardiovascular risk.(5)

The name NAFLD fails to capture the metabolic component of the disease, which is crucial for disease progression. A new terminology – metabolicdysfunction associated steatotic liver disease (MASLD) - has been proposed to reflect the growing recognition of the underlying metabolic dysfunction. This new entity now encompasses, by definition, the presence of a cardiometabolic risk factor in addition to the traditional NAFLD criteria. (6)

In the long term, the natural history of NAFLD shows that the mortality of these patients is primarily due to cardiovascular causes, then neoplasms and secondarily to hepatic causes, namely cirrhosis and hepatocellular carcinoma.(7) Liver fibrosis assessed noninvasively is an independent predictor of mortality (especially from cardiovascular causes) in patients with NAFLD.(8)

The use of scores combining biochemical and clinical data, together with the results of transient elastography using the Fibroscan device, is a practical alternative to liver biopsy for the assessment of liver damage.(9)

Non-alcoholic fatty liver and cardiovascular disease share common risk factors, with several studies establishing NAFLD as an independent CV risk factor. In patients with NAFLD cardiovascular disease is the most common cause of death.(10) The newly adopted terminology for NAFLD-metabolic dysfunction associatedsteatotic liver disease - underscores its systemic and dysmetabolic nature, which transcends beyond hepatic involvement.(6) Individuals with MASLD have a combination of metabolic syndrome risk factors and carotid atherosclerosis. Recent studies also reveal a significant link between MASLD and increased arterial stiffness, suggesting a common pathogenesis.(11) Arterial stiffness is a significant independent risk factor

for a number of cardiovascular diseases, including hypertension, heart failure, stroke and myocardial infarction.

In Romania, there are not enough studies investigating the correlation between the components of the metabolic syndrome and the degree of hepatic fibrosis and steatosis assessed by noninvasive methods - which is what the present study aims to achieve. We used machine learning techniques (Classification and Regression Tree - CART) to classify patients with steatosis and liver fibrosis based on clinical parameters and metabolic syndrome components. Using the CART algorithm, we identified patients at high risk of liver disease progression, who may benefit from further monitoring with transient elastography (TE).

Another aspect addressed in this study, is the cardiovascular risk that the presence of NAFLD entails, initially through subclinical changes of atherosclerosis. The identification of subclinical atherosclerosis draws attention to the risk of cardiovascular morbidity and mortality, even in patients with asymptomatic liver disease.

Detection of atherosclerosis in patients with NAFLD can be done noninvasively using carotid ultrasound and the Tensiomed arteriograph, measuring parameters such as carotid intima media thickness (CIMT), augmentation index (Aix) and pulse wave velocity (PWV) to assess arterial stiffness. The research focuses on studying the correlation between hepatic steatosis,fibrosis, and markers of atherosclerosis. We used machine learning techniques like cluster analysis, in order to understand how metabolic syndrome, liver damage and arterial stiffness interact.

These results are clinicallysignificantand support the need for a more comprehensive diagnostic approach when metabolic syndrome or non-alcoholic fatty liver disease is suspected.

The third study presents the correlation between cardiovascular risk - calculated using the Systematic COronary Risk Evaluation 2 (SCORE2) chart, and noninvasive tests for liver fibrosis and steatosis, as well as markers of systemic atherosclerosis. Cardiovascular risk calculators have limitations that the direct noninvasive measures we use can avoid. The noninvasive approach provides information on arterial wall structure in people of all ages, using a continuous measure to describe progression or regression of atherosclerosis at all stages.

There is no specific pharmacological therapy for non-alcoholic steatohepatitis, lifestyle modification through healthy eating and physical activity is the main recommendation.(12) People with NAFLD often exhibit prolonged sedentary behaviours and poor nutrition.(13)

Engaging in physical activity in adults has positive effects on health, including reductions in all-cause mortality, cardiovascular disease mortality, hypertension, type 2 diabetes, certain cancers, and adiposity. To achieve these benefits, the World Health Organization recommends that all adults engage in regular physical activity.(14) Studies attest to the effectiveness of physical activity in reducing intrahepatic fat content, independent of weight loss.(15) In order to effectively assess the degree of physical activity and to provide tailored recommendations to patients, a simple, standardised and validated tool is needed. The Physical Activity Index (PAI) classifies patients as active, moderately active, moderately inactive or inactive. Activity level correlates with cardiovascular risk.(16) In the third study we investigated the correlations between the extent of physical activity (assessed by PAI) and the number of metabolic syndrome components and found an inverse correlation between them.

The results of these studies show that the degree of liver damage correlates with the severity of the metabolic syndrome and of systemic atherosclerosis. Moreover, the level of physical activity is inversely related to the number of cardiometabolic risk factors. This toolcan be used to assess cardiovascular risk and institute therapeutic measures to prevent cardiovascular events.

GENERAL SECTION

The first three chapters of the thesis contain a theoretical exposition of non-alcoholic fatty liver, metabolic syndrome and atherosclerosis, as well as a presentation of the noninvasive assessment modalities for these entities.

In chapter four, we reviewed the existing literature to describe the common pathogenic mechanisms and their interaction, in order to show how these conditions are linked. We described the contribution of each cardiometabolic factor (abdominal obesity, high blood pressure, elevated triglycerides, low HDL cholesterol, diabetes mellitus or elevated blood glucose) to the liver and vascular damage, demonstrating the connection between these pathologies.

SPECIAL SECTION

Study 1. Exploring the correlation between metabolic syndrome components and non-alcoholic fatty liver disease

1.1 Introduction and working hypothesis

Our hypothesis is that there is a correlation between liver damage and metabolic syndrome components and that these can be assessed noninvasively.

In this study, we aimed to quantify the extent of hepatic steatosis and degree of fibrosis using noninvasive markers in patients with suspected MASLD, taking into account the presence of metabolic syndrome components. Using two-step cluster analysis machine learning techniques, we stratified patients with hepatic steatosis and fibrosis based on clinical indicators and metabolic syndrome components to identify those at risk of liver disease progression who would benefit from transient elastography (TE) and subsequent closer monitoring. We used a Classification and Regression Tree (CART) algorithm, which is valuable because of its adaptability to different types of data and distributions, robustness against outliers, and efficient handling of missing data through automatic alternative splits. The algorithm identifies optimal split points (threshold values) for predictor variables, demonstrating adaptability in practical applications.(17)

Combining clinical and biological data in machine learning algorithms improves prediction accuracy and represents an advanced approach in line with the principles of precision medicine.

1.2 Study objectives

- obtaining a general biochemical profile of patients, including liver function tests
- assessment of noninvasive serological and imaging markers of steatosis and liver fibrosis
- establishing correlations between the components of the metabolic syndrome and the degree of fibrosis and hepatic steatosis

- grouping patients according to risk of liver damage using machine learning techniques

1.3 Material and method

We conducted an analytical, observational, prospective study,in 217 patients who were admitted between January 2021 and January 2023 to the medical wards of the Sibiu County Emergency Hospital (SCJUS). Each patient signed an informed consent form to participate and the research was conducted with the approval of the Ethics Committee of the SCJUS. Patients were referred for further evaluation in our centre after suspicion of MASLD was raised in different centres due to the presence of hepatic steatosis on abdominal ultrasound. Data collection began before the new nomenclature for MASLD was established and, accordingly, we used the inclusion criteria that defined NAFLD. The process of conducting the research is represented graphically in Figure 1.

Those with alcoholic hepatitis were excluded from the study, as well as those with liver disease attributable to other aetiology.Patients were included based on self-reported lack of significant alcohol consumption (defined as <20 g/day for women and <30 g/day for men).(18)

Therefore, subjects diagnosed with NAFLD, as previously defined, and patients who, according to the new nomenclature for fatty liver disease, fell into either the MASLD or possible MASLD/cryptogenic steatotic liver disease category were included. The latter applies to patients with a normal BMI in whom metabolic syndrome components were not identified.

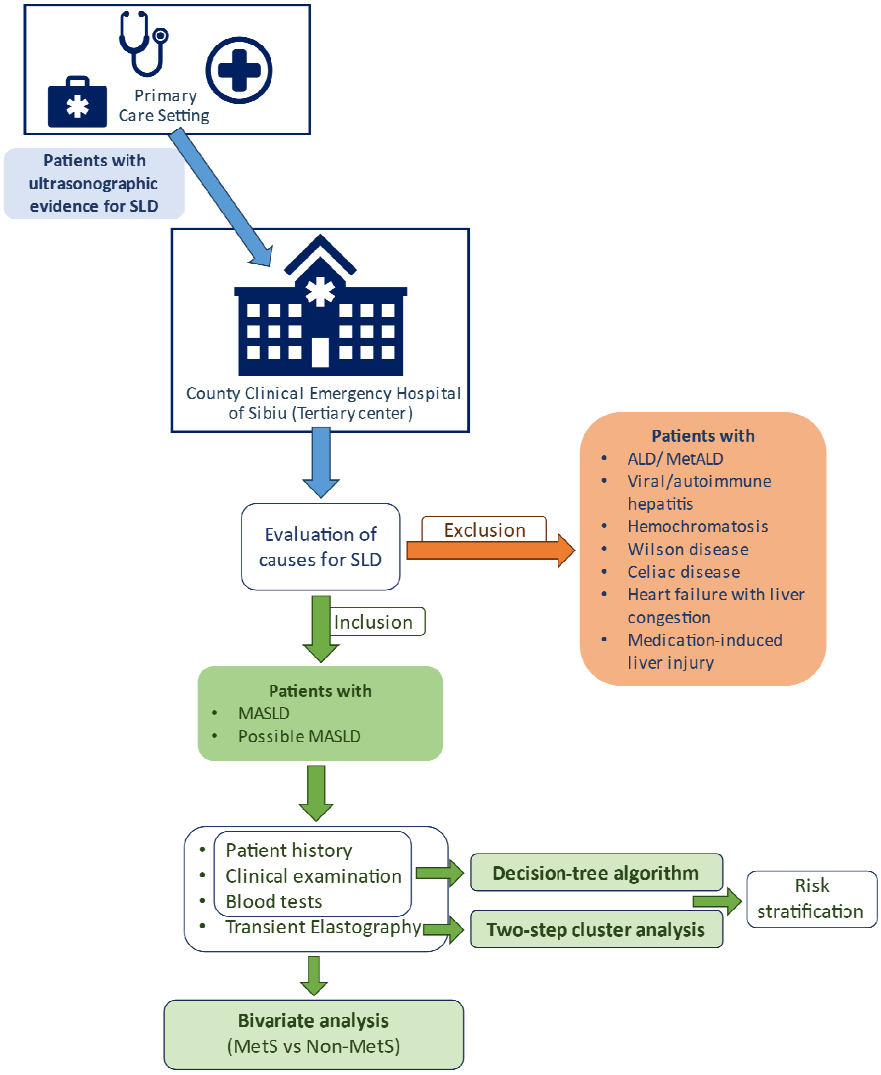

Figure 1. Study workflow

Each patient underwent clinical and paraclinical examinations (including TE) during the same visit. Medicalhistory, age, gender, height, weight, body mass index (BMI), waist circumference, blood pressure, heart rate, full blood count, aspartate aminotransferase (AST), alanyl aminotransferase (ALT), triglycerides (TGL), blood glucose, liver stiffness (LSM) and hepatic steatosis (CAP) were recorded.

The diagnostic criteria for metabolic syndrome (minimum 3 out of 5) were (19): waist circumference ≥ 94/80 cm for men/women; blood pressure ≥ 130/85 mm Hg or treatment for hypertension; fasting blood glucose ≥ 100 mg/dl or treatment for type 2 diabetes; serum triglycerides > 150 mg/dl; HDL cholesterol < 40/50 mg/dl for

men/women. The number of metabolic components present in each patient (0-5) was noted.

The serological and clinical data obtained were used to calculate the AST/ALT ratio (AAR), with a threshold value <0.8 to exclude fibrosis(20); APRI - a value of 0.5 can be used to exclude significant fibrosis (21); FIB4 - a value <1.3 excludes significant fibrosis(22) and hepatic steatosis index (HSI). Values below 30 exclude NAFLD and values above 36 detect NAFLD.(23)

The FibroScan 502 Touch was used to measure liver stiffness (E or LSM) and steatosis (CAP) by transient elastography, in compliance with the quality criteria required by the manufacturer. Steatosis was classified into four categories (S0-S3) using the limits provided by the meta-analysis performed by Karlas T. et al.(24). Fibrosis measured using TE (LSM or E) divided patients into five groups (F0-F4), with reference values based on a meta-analysis of studies comparing transient elastography and liver biopsy results.(25)

We used data obtained by TE and other variables (AST, ALT, platelets, age, gender, presence of type 2 diabetes) to calculate Agile3+ and Agile4 scores. These represent the probability of significant fibrosis, i.e. cirrhosis, in patients with NAFLD. Since fibrosis is estimated by a logistic regression model, it ranges from 0 to 1 and can be interpreted probabilistically.(26)

Statistical analysis

The data were analysed with IBM SPSS Statistics 21. Continuous variables were characterized by means, medians, standard deviation and others. Categorical variables were analyzed by frequency distributions. Statistical tests such as t-Student, Mann-Whitney and chi-square tests were used to assess the significance of the results, with $p < 0.05$ considered as significance level.

Application of machine learning techniques

Cluster analysis involves grouping subjects into categories or clusters based on their similarities and differencesin certain characteristics. The aim is to form homogeneous and distinct groups in order to better understand variation and similarities among subjects. Each subject is assigned to a single category or cluster, and these are how the research data are structured. Next, we implemented a CART algorithm to explore a set of discriminant rules and distinguish between the defined

clusters. Decision trees are supervised machine learning methods that categorize data, thus revealing hidden patterns behind user-defined outcomes.(27) This leads to the creation of a graphical decision model centered on the target variable. We provided the following continuous variables to the algorithm: age, BMI, ALT, AST, platelet count, and three of the five dichotomous metabolic syndrome characteristics: hypertriglyceridemia or treatment for dyslipidemia, low HDL cholesterol or treatment for dyslipidemia, and fasting glucose ≥ 100 mg/dl or treatment for type 2 diabetes.

1.4 Results

The study included 217 patients with suspected MASLD, aged 19 to 85 years, of whom 126 were female and 91 were male. Of these, 109 met the criteria for metabolic syndrome (MS).

Variables studied in the two groups included age, body mass index (BMI), AST, ALT, platelets, CAP, E, APRI, FIB-4, AAR, Agile 3+ and 4 scores, and HSI. All variables except AAR showed statistically significant association with MS.

Women in the study had higher BMI, higher platelet counts, higher AAR and HSI, and lower ALT and APRI compared to men. In terms of gender distribution by MS, it was similar between the groups with and without MS. The metabolic risk factor present most frequently in the MS patients was high blood glucose/type 2 diabetes, followed by hypertension and increased abdominal circumference.

According to the new nomenclature for steatotic liver disease, 91.71% of patients included met the criteria for the diagnosis of MASLD. A total of 18 patients (8.29%) had no cardiometabolic component associated with steatotic liver disease and were therefore classified as having cryptogenic steatotic liver disease.

In terms of analysis of steatosis and liver fibrosis, only 8.25% of MS patients were free of steatosis, and severe steatosis (S3) was more common in those with MS. Regarding fibrosis, most patients without MS did not have significant fibrosis, while those with MS had significant fibrosis and cirrhosis in higher proportions.

For risk stratification, an algorithm based on CAP and E values was used, which identified three clusters. Cluster 1 defined patients at low risk of liver damage, while those in cluster 3 had a higher risk of significant liver fibrosis and steatosis. Cluster 2 included patients with at least moderate steatosis. Of the 18 patients without cardiometabolic criteria, 17 were included in cluster 1.

Combining clusters 2 and 3 resulted in a subpopulation at increased risk of liver damage. The resulting data are presented in Tables 1 and 2.

Table 1. Fibrosis grades in the two risk groups

Variable	**Feature**	**Cluster 1**	**Cluster 2&3**	***p***
Number	-	93	124	-
Fibrosis category	F0	71 (76.3%)	71 (57.3%)	< 0.01
	F1	10 (10.8%)	7 (5.6%)	
	F2	7 (7.5%)	16 (12.9%)	
	F3	4 (4.3%)	17 (13.7%)	
	F4	1 (1.1%)	13 (10.5%)	

Table 2. Steatosis grades in the two risk groups

Variable	**Feature**	**Cluster 1**	**Cluster 2&3**	***p***
Number	-	93	124	-
Steatosis categories	S0	38 (40.9%)	7 (5.6%)	< 0.01
	S1	28 (30.1%)	11 (8.9%)	
	S2	12 (12.9%)	38 (30.6%)	
	S3	15 (16.1%)	68 (54.8%)	

We performed a CART decision tree algorithm to identify relevant predictors for risk categorization (cluster 2&3).

Figure 2 shows the rules within the algorithm as well as the resulting final nodes.

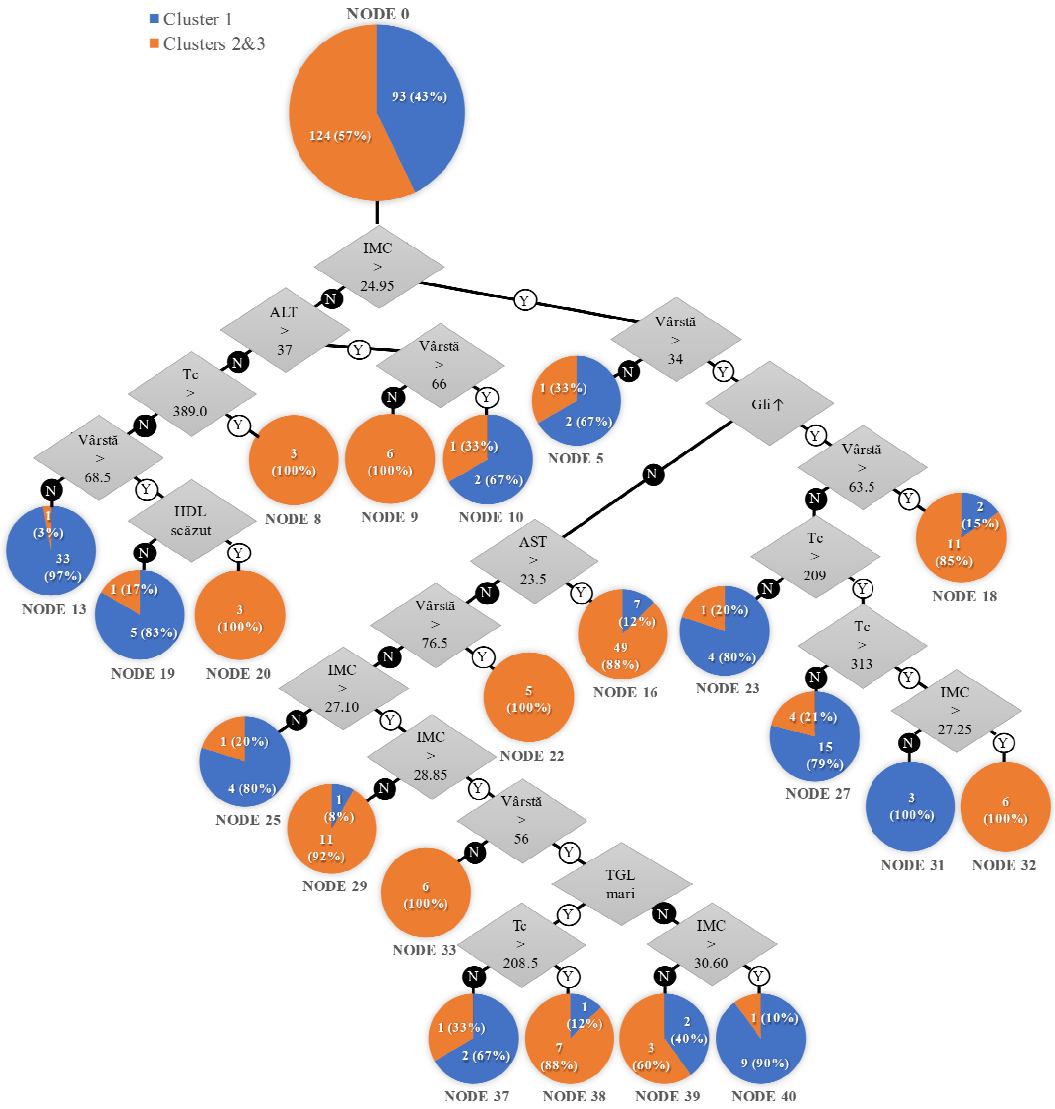

Figure 2. Decision tree for cluster membership prediction

Where node-node, Y-Da, N-Nu, BMI-body mass index, Tc-thrombocytes, Tgl-triglycerides, gli-glycemia.

The resulting model predicts cluster membership 1 with 86% accuracy and combined cluster membership 2 and 3 with 91.9% accuracy. The CART algorithm identifies distinct risk categories for liver disease assessed by TE. Of the twenty-one endpoints, the majority indicate risks below 33% or above 85%, with the exception of one endpoint (37) indicating a 60% risk. This shows the effectiveness of the algorithm in stratifying individuals at risk of liver disease.

In the CART model, BMI over 24.95 kg/m² is the first rule for stratifying patients. Based on criteria such as BMI, age, ALT level, HDL cholesterol, diabetes, blood glucose, triglycerides and platelet count, patients are divided into different main groups and subgroups according to their individual characteristics. Figure 3 illustrates the various subpopulations within the high-risk group based on their defining characteristics as determined by the CART algorithm.

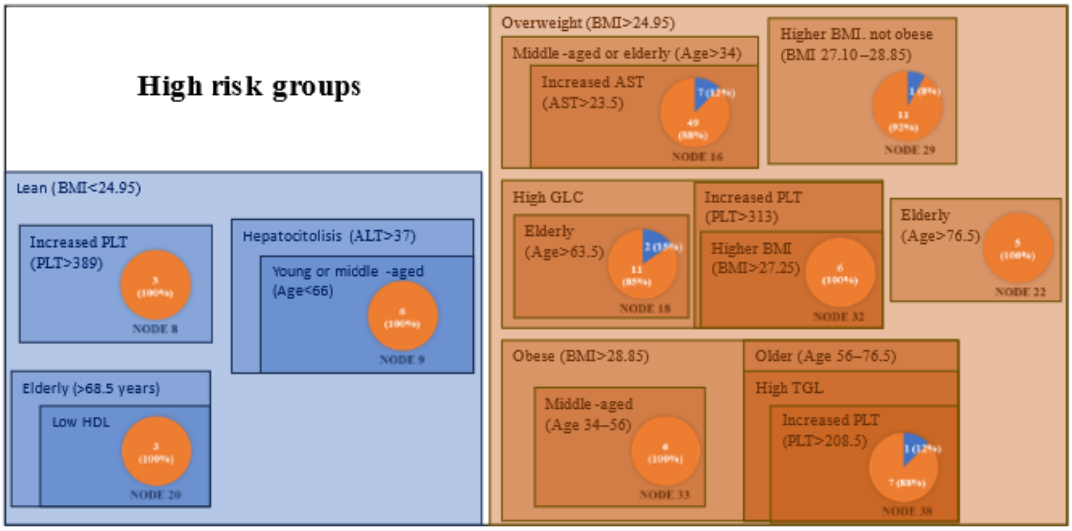

Figure 3. Subpopulations at high risk of liver disease identified by the CART algorithm

1.5 Discussion

Patients with MetS were older and had higher AST and ALT levels compared to those without MetS. Body mass index was higher in the MetS group. Assessment of steatosis (CAP) and fibrosis (E) showed significant differences between the two groups.

Using the decision tree algorithm, specific patient profiles were identified. Normal BMI acted as a protective factor and BMI had an increased predictive value in younger patients. Impaired glucose tolerance was an important factor in patient classification. Younger patients had advanced MASLD if they were obese, while older patients required further stratification according to metabolic risk factors and platelet count.

The resulting model had an accuracy of 89.4% in terms of predictability of cluster membership, supporting the use of the two step cluster analysis technique in selectingpatients for further investigations (blood tests, serological scores, followed

by TE). The results highlight the link between MetS and MASLD and support the adoption of the new terminology and the use of noninvasive tests to identify those at risk of advanced liver disease.

Study 2. Study of correlations between liver damage and systemic atherosclerosis using noninvasive markers

2.1 Introduction and working hypothesis

People with MASLD have a combination of risk factors for metabolic syndrome and carotid atherosclerosis. This diagnosis should signal the presence of increased cardiovascular risk. Arterial stiffness becomes more pronounced in various cardiovascular and metabolic diseases, and its progression appears to occur before obvious target organ damage. This implies that increased arterial stiffness may play a central role in the link between cardiovascular risk and cardiovascular events, making it a potential target for preventive and therapeutic strategies. The European Society of Cardiology guidelines for the treatment of hypertension have recommended the use of pulse wave velocity (PWV) measurements to assess arterial stiffness.(28)

In this study we used the TensioMed™ arteriograph, which uses an oscillometric technique to estimate PWV. Pulse wave velocity (PWV) provides information about aortic stiffness and is a strong predictor of major cardiovascular events.(29) Pulse wave analysis can also highlight endothelial dysfunction by measuring augmentation index (Aix) and central systolic blood pressure (SBPAo).

In this research, we intend to investigate the impact on liver and arterial stiffness in the context of metabolic syndrome using noninvasive methods (such as serological scores and transient elastography for liver assessment, and PWV and carotid intima-media thickness -CIMT measurement for arterial stiffness). Our starting hypothesis assumes a correlation between atherosclerosis and liver damage in NAFLD, with this damage being more pronounced with the presence of additional metabolic factors.

2.2 Study objectives

- a general biochemical profile of patients, including liver function tests
- assessment of noninvasive serological and imaging markers of steatosis and liver fibrosis
- assessment of noninvasive markers of atherosclerosis - arterial stiffness (PWV), endothelial dysfunction (Aix), carotid intima-media thickness (CIMT)

- study correlations between liver damage and systemic atherosclerosis using measured parameters
- applying machine learning techniques to identify subpopulations with common characteristics

2.3 Material and method

We conducted an observational, prospective study in a cohort of 75 patients (30 men and 45 women), who were referred for further investigations to the Sibiu County Emergency Clinical Hospital between May 2021 and May 2023, after presenting with either hepatic steatosis on abdominal ultrasound, changes in liver function tests, or the presence of cardiometabolic risk factors. All patients were clinically evaluated and underwent laboratory tests, abdominal and carotid ultrasound (General Electric S8), liver transient elastography (with the Fibroscan 502 Touch device) and evaluation with the Tensiomed arteriograph (produced by Medexpert, Budapest, Software version 3.0.0.3). The study was approved by the SCJUS Ethics Committee and participants provided informed consent. Patients with liver pathology other than NAFLD were excluded.

Similar to the methodology of the first study we obtained the following variables: age, body mass index (BMI), AST, ALT, platelets (Tc), serum triglycerides (TGL), HDL cholesterol, CAP (dB/m), E (kPa), APRI, FIB 4, AAR, Agile 3+ score, Agile 4 score and HSI.

The TensioMed Arteriograph records oscillometric pressure curves using plethysmography, capturing pressure changes in an artery via a cuff applied to the arm. By analysing these changes, the arteriographer calculates the time difference between the first wave and the reflected wave, thus estimating PWV. Reference values for PWV are 5-15 m/s, and can vary according to age and blood pressure levels. Increased pulse wave velocity is associated with reduced arterial compliance and increased arterial stiffness.(30,31)

The parameters assessed using TensioMed were the aortic augmentation index (AoAix) to assess endothelial dysfunction and pulse wave velocity (PWV) to assess aortic wall stiffness.

B-mode carotid ultrasound was performed using a linear transducer with a frequency of 8 MHz. Carotid intima-media thickness (CIMT) was measured along an arterial segment without atherosclerotic plaques. A straight arterial segment of 10 mm length was selected, with measurements taken along the far wall of the common carotid artery.

Statistical analysis

Data analysis included the calculation of statistics for continuous variables and frequency distributions for categorical variables. For continuous variables, t-student or Mann-Whitney U tests were used, depending on the data distribution, and ANOVA or Kruskal-Wallis for between-group comparisons. Associations between categorical variables were assessed with the chi-square test or Fisher's test. Statistical significance was considered at $p < 0.05$.

Application of machine learning techniques

We used the "two step cluster analysis" technique to group observations based on common characteristics, including parameters from TE and arterial stiffness measurements. The optimal number of clusters was determined using the Akaike Information Criterion (AIC), with an average cohesive silhouette value above 0.5 to indicate good model quality.

2.4 Results

The study included 75 patients (30 men and 44 women). There were no significant gender differences in age, BMI, number of MetS elements, HDL cholesterol, triglycerides, AST, FIB4, AAR, APRI, E (kPa), CAP (dB/m), AGILE3+, AGILE4, PWV, AoAix and CIMT.

Age, HDL cholesterol, HSI, PWV, AoAix and CIMT had a normal distribution between genders. Regarding categorical variables, there were no differences for the presence of MetS, type 2 diabetes (T2DM), elevated blood glucose, elevated waist circumference, elevated triglycerides, low HDL cholesterol, hypertension, BMI category, APRI category, FIB4, AAR or HSI.

The number of patients who met the criteria for metabolic syndrome was 26 out of 75. Age, HDL cholesterol, Tc, CAP, AoAix, CIMT and HIS had a normal distribution according to the MetS categories. 47 patients (19 males and 28 females)

were diagnosed with MASLD, while 16 had no steatosis or fibrosis in TE. No significant differences were found between the two groups in platelet counts, ALT, AST, HDL cholesterol, APRI and AAR values.

The general details of the clustering model and the description of the resulting clusters are presented in Table 3.

Table 3. Overview of the clustering model

Variable	Feature	Cluster 1	Cluster 2	Cluster 3	Meaning p
Number	-	28 (37.3%)	13 (17,3%)	34 (45.3%)	
CAP (dB/m)	Medium	226.46	246.23	319.58	<0.01
	DevStd	39.33	36.57	39.33	
	IQR	58.25	33.5	45.25	
	MIN	140	198	271	
	MAX	300	336	381	
	95%CI	211.21 - 241.71	224.13 - 268.33	309.81 - 326.37	
	The importance of the predictor		1		
PWV	Medium	7.38	12.23	9.46	<0.01
	DevStd	1.43	2.02	1.2	
	IQR	2.03	2.2	1.78	
	MIN	4.1	9.8	7.6	
	MAX	10.1	17.7	12	
	95%CI	6.83 - 7.94	11 - 13.45	9.05 - 9.88	
	The importance of the predictor		0.88		

Cluster analysis was performed using variables obtained by TE and arterial stiffness measurements (PWV, CAP). Cluster 1 showed significantly lower values for both parameters compared to cluster 3 and significantly lower values for PWV compared to cluster 2.

The frequency of metabolic syndrome, as well as individual frequencies of increased abdominal circumference, increased fasting glucose or type 2 diabetes, and hypertension as defining features of MS, were significantly more common in cluster 3, followed by cluster 2 and less common in cluster 1. Low HDL levels were also more common in clusters 2 and 3 compared to cluster 1. Although the categorical variable defining high triglycerides (above 150 mg/dl) did not show significant differences between clusters, absolute serum triglyceride values were significantly higher in cluster 3 followed by cluster 2 and lower in cluster 1.

2.5 Discussion

Patients with metabolic syndrome showed more signs of liver damage, including increased HSI, FIB4, and AGILE3+ and AGILE4 scores, as well as higher E and CAP values. However, no significant correlations were found between AST, ALT, platelet count and metabolic syndrome, although the APRI category showed a significant correlation with its presence. The results suggest the influence of metabolic syndrome on liver impairment and arterial stiffness, evidenced by PWV and CIMT, in the context of NAFLD. PWV, AoAix and CIMT showed significantly higher values in patients with MS. These correlations have been well documented in the literature, as well as their association with liver damage in MetS.(32) A study that aimed to assess the connection between NAFLD and progression of arterial stiffness (PWV) showed that those with NAFLD had a more rapid progression of arterial stiffness over time, regardless of other cardiovascular risk factors.(33)

In our study, we identified three distinct clusters. Cluster 3 showed the highest values of hepatic steatosis and arterial stiffness and was associated with metabolic syndrome. Cluster 2 had increased arterial stiffness but no significant hepatic steatosis, and Cluster 1 was the reference group with the lowest values for both aspects. This suggests that hepatic steatosis, arterial stiffness and metabolic syndrome tend to manifest together as the severity of metabolic dysfunction increases. Research suggests that hepatic steatosis is an independent risk factor for subclinical atherosclerosis.

Study 3. Correlations between cardiovascular risk assessment using the SCORE2 diagram and noninvasivemarkers of atherosclerosis and liver damage

1.1 Introduction

The lifetime risk of cardiovascular events is around 50% for people over 30, even in the absence of a known history of CVD.(34) MetS, NAFLD and subclinical atherosclerosis are interconnected conditions associated with cardiovascular disease. Although models used to predict cardiovascular risk are widespread, they have limitations. A significant proportion of cardiovascular events occur in individuals who are classified as low or intermediate risk according to these models. This limitation has sparked interest in additional tools such as carotid intima media thickness (CIMT) measurement and pulse wave velocity (PWV) assessment to improve cardiovascular risk identification.(35)

In Romania, one of the ways of assessing cardiovascular risk is to use the SCORE2 (Systematic COronary Risk Evaluation 2) chart, which aims to predict the 10-year risk for both fatal and non-fatal cardiovascular events in people aged 40-69. The SCORE2 risk models are tailored to take into account the cardiovascular mortality rate at country level, classifying the countries into four categories: low, moderate, high and very high, based on standardised cardiovascular disease mortality rates. Romania falls into the very high risk category because of its high cardiovascular mortality rate. European SCORE2 models take into account traditional cardiovascular risk factors such as age, gender, smoking history, presence of diabetes, blood pressure and cholesterol levels.(36) SCORE2 charts have been integrated into the 2021 European Society of Cardiology (ESC) Guidelines on cardiovascular disease prevention in clinical practice.(37)

The present study assessed the correlation between risk classifications obtained using the SCORE2 chart and noninvasive parameters obtained by assessing the liver (LSM, CAP), and atherosclerosis (PWV, CIMT) in a cohort of 41 patients aged 40-69 years.

1.2 Objectives

The aim of the research was to assess the extent to which noninvasive parameters, such as arterial stiffness, carotid intima-media thickness and

noninvasiveliver assessment tests, can be used to improve the accuracy of cardiovascular risk assessment in patients with metabolic syndrome.

1.3 Material and method

We conducted an observational study covering the period from June 2021 to April 2023 in a cohort of 41 patients who requested medical evaluation at the Sibiu Emergency Clinical Hospital. Reasons for seeking medical care included the presence of metabolic risk factors, increased liver echogenicity on ultrasound suggestive of steatosis, or abnormal blood test results for liver assessment. We meticulously obtained the Ethics Committee approval and informed consent from all participants. The clinical and paraclinical evaluation was similar to the one described in the previous study. Patients with liver disease other than MASLD were excluded. In this study we included only patients aged 40 to 69 years, as the SCORE2 chart was validated for this age group.

Liver damage was assessed both by Fibroscan and by serological scores. Arterial stiffness was measured using the Tensiomed arteriograph, while CIMT was quantified sonographically. Cardiometabolic risk factors were also recorded.

We used the SCORE2 chart to calculate cardiovascular risk for each patient, taking into account age, gender, smoking status, systolic blood pressure and cholesterol level. The estimated score reflected the probability of suffering a fatal or non-fatal cardiovascular event in the next 10 years, with patients classified into three risk categories: low to moderate risk, high risk and very high risk.

1.4 Results

Of the 41 patients (16 men and 25 women), 10 met the criteria for MetS, with 28 patients classified as very high cardiovascular risk according to SCORE2.

No statistically significant differences were found between genders in age, PWV, CAP, CIMT, E (kPa), APRI, FIB-4, triglycerides (TGL), HDL, SCORE2 category (very high risk vs. other category), fibrosis category, CAP grade, APRI category, FIB4 category or AAR category. There were statistically significant gender differences in HDL cholesterol, with higher values in women, as shown in Table 4.

Table 4. Gender differences in HDL cholesterol values

Variable	Descriptive parameter	Gen		Meaning p
		Female	Male	
HDL	Medium	69.92	55.37	<0.01
	DevStd	16.7	12.72	
	IQR	31	22.75	
	MIN	39	38	
	MAX	100	80	
	95%CI	63.03-76.81	48.6-62.15	

Compared to patients without MS, those with MS had a higher degree of steatosis (CAP 319.1 versus 251.94 dB/m), fibrosis (E 9.43 versus 4.96 kPa) and a greater carotid intima-media thickness (CIMT) (1.04 versus 0.79 mm).

The category of liver fibrosis assessed by Fibroscan or FIB4- score was higher in those with MetS compared to those without MetS. Fibroscan assessment did not identify fibrosis in 90.3% of patients without MetS, and FIB-4 score excluded fibrosis in 87.1% of these patients (FIB-4 values<1.3). Patients with MetS had significantly more advanced liver fibrosis.

Arterial parameters (AixAo, PWV, CIMT) showed significantly worsevalues in those classified as very high risk according to SCORE2. Pulse wave velocity (PWV) was higher (10.07 vs 8.22 m/s), indicating increased arterial stiffness. Liver stiffness measured by elastography (LSM) or FIB-4 score showed higher values in those in the very high risk category according to SCORE2.

3.6 Discussion

The results of this study showed that patients with metabolic syndrome had higher levels of arterial stiffness, CIMT and liver stiffness. A significant correlation was also found between these parameters and the increased cardiovascular risk category determined by the SCORE2 diagram. This indicates that patients with metabolic syndrome are more prone to subclinical cardiovascular injury and that assessment of these noninvasive parameters may provide valuable information for stratifying their cardiovascular risk.

The observed difference in the lower rate of cardiovascular disease in women compared to men is well known in the literature.(38) The higher HDL cholesterol levels found in women in our study could contribute to this difference and could be

part of the explanation for the lower cardiovascular risk observed in women. Women may have a lower cardiovascular risk due to a combination of factors, higher HDL cholesterol levels representing only part of the equation.

The SCORE2 chart and our arterial and liver stiffness assessment methods showed good agreement in classifying patients into risk categories. However, our study promotes the adoption of noninvasive techniques as superior alternatives to conventional cardiovascular risk prediction models. The reason for this change stems from the notable limitations observed in current risk models. While effective in identifying high-risk individuals, these models may neglect those with more marginal risk factors, leading to false reassurance for some and missed opportunities for intervention.(39)

Study 4. Physical activity in patients with metabolic syndrome and NAFLD

4.1 Introduction

In the absence of pharmacological treatment, dietary interventions and exercise are fundamental treatments for NAFLD, addressing sedentary behaviours and poor nutrition that contribute to the pathogenesis of the condition. (13) Regular physical activity can improve key components of metabolic syndrome and reduce fat content in the liver, independent of weight loss.(12,15,40). The World Health Organization recommends that adults engage in regular physical activity to improve health. A useful tool for assessing physical activity levels is the PAI (physical activity index) questionnaire. For those who are inactive, interventions to improve activity levels are recommended.

This study examines the physical activity levels of adults aged 18 to 74 diagnosed with NAFLD or MetS.

4.2 Objectives

The objectives of our study were to identify possible correlations between physical activity level (assessed by PAI) and the number of MetS components. Also, a secondary objective was to identify significant differences in physical activity levels between MetS and non-MetS patients.

4.3 Material and method

We conducted an observational, prospective study between January 2021 and January 2023 at the Sibiu Emergency Hospital. The study was approved by the Ethics Committee and participants provided informed consent. Of the entire cohort, in 39 patients we did not identify hepatic steatosis by TE. Subsequently, two distinct groups were formed from the remaining patients: 90 people (36 men and 54 women) met the criteria for the diagnosis of MetS, while 71 (31 men and 40 women) were diagnosed with NAFLD but without metabolic syndrome. Exclusion criteria were the same as in previous studies.

In the entire cohort of patients examined (200 people), who underwent Fibroscan assessments and the PAI questionnaire, we performed an analysis to establish correlations between the PAI score and various parameters. These parameters included the number of metabolic syndrome risk components, age,

abdominal circumference, triglyceride and HDL levels, blood pressure values, presence or absence of diabetes diagnosis, AST, ALT, platelet count, body mass index, elastographic values- liver stiffness- LSM (kPa) and steatosis-CAP (dB/m) by Fibroscan, height, weight, APRI score and FIB 4 score.

Participants completed a short questionnaire (completion time approximately one minute) that assessed work-related physical activity, leisure time activities and walking pace. The results of the questionnaire were instantly calculated using an online Excel version (41), which placed them into one of four activity categories: active (0), moderately active (1), moderately inactive (2) and inactive (3).

The interpretation of the questionnaire results is as shown in Table 5.

Table 5. Interpretation of the physical activity questionnaire

INACTIV	sedentary work and no exercise or cycling
MODERATELY INACTIVE	sedentary work and less than one hour of exercise and/or cycling per week OR standing work and no exercise or cycling
MODERAT ACTIV	sedentary work and 1-2.9 hours of exercise and/or cycling per week OR standing work and less than one hour of exercise and/or cycling per week OR physical work and no exercise and/or cycling
ACTIV	sedentary work and more than 3 hours of exercise and/or cycling per week OR standing work and 1-2.9 hours of exercise and/or cycling per week OR physical work and less than 1 hour of exercise and/or cycling per week OR heavy physical work

The results were statistically analysed using the Student's t-test and Pearson correlation index. The Pearson correlation measures the linear relationship between variables, ranging from -1 to 1, where -1 indicates an overall negative correlation, 0 indicates no correlation, and 1 indicates an overall positive correlation.

4.4 Results

Patients with MS had a higher mean age, higher AST and ALT levels, and a higher BMI compared to those without MetS. Also, assessment of steatosis and liver fibrosis showed significant differences between the two groups. Significant correlations were observed between the presence of metabolic syndrome and physical activity level ($r = 0.749$). However, liver enzymes (AST and ALT)did not show significant correlations with physical activity. Other significant correlations were observed between physical activity and waist circumference, triglyceride levels, HDL, blood pressure, presence of diabetes, body mass index and liver stiffness (LSM,

APRI, FIB4). Patients with metabolic syndrome had significantly higher liver stiffness and fibrosis scores (APRI and FIB4) compared to those without metabolic syndrome.

Comparing the two groups in terms of physical activity index, we found that patients with MetS were less active than those without MetS, the difference being statistically significant ($p < 0.05$) (Figure 4).

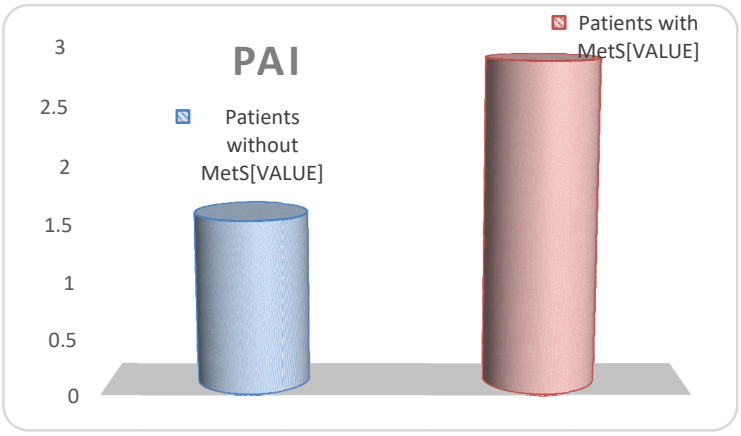

Figure 4. Physical activity index in patients without MetS vs. with MetS

4.5 Discussion

In this study, physical activity levels were compared in groups of patients with MetS and NAFLD without MetS. People with MetS were found to have more severe steatosis and liver fibrosis. Body mass index was also higher in the MetS group. Age was also higher in the MetS group, and the higher presence of women in this group may be attributed, in part, to hormonal changes associated with menopause that may contribute to weight gain and metabolic changes.(42)

AST and ALT values showed variations between groups, with increased ALT levels in MetS patients, which may suggest the presence of steatohepatitis. We also found significant correlations between physical inactivity and various indicators of metabolic syndrome, highlighting the importance of an active lifestyle in the treatment and prevention of these conditions.

FINAL CONCLUSIONS

1. Patients with MS had significantly higher values for age, BMI, hepatic steatosis index (HSI),FIB-4, AGILE3+ and AGILE4 scores, and E(kPa) and CAP(dB/m), indicating an increased prevalence of liver damage in this cohort.

2. According to the new nomenclature for steatotic liver disease, 91.71% of the patients included in the study met the criteria for the diagnosis of MASLD, and 18 patients (8.29%) had no cardiometabolic component associated with steatotic liver disease.

3. The most common metabolic risk factor found in the study population was high fasting blood glucose/type 2 diabetes mellitus, 65.44% in the group without MetS and 88.99% in the group with MetS.

4. Using cluster analysis we identified three categories of patients: cluster 1 with low risk of liver disease, cluster 2 with higher chance of moderate steatosis, and cluster 3 with predisposition to significant fibrosis and steatosis. Combining clusters 2 and 3, we created a subpopulation with increased risk of liver damage. Cluster 2, which included patients with more severe hepatic steatosis, was characterized by CAP values of at least 276 dB/m, in accordance with EASL guidelines for the detection of hepatic steatosis.

5. Applying the CART algorithm, we identified age, BMI, transaminase levels and metabolic syndrome characteristics as significant stratification factors. The first predictive factor for the risk categories was BMI, with a baseline value above 24.95 kg/m^2. Normal BMI acted as a protective factor, except for certain subgroups of patients (young with high ALT, elderly with low HDL cholesterol).

6. PWV, AoAix and CIMT values were significantly higher in MetS patients, confirming previous findings in the literature about the link between MetS and arterial rigidity. The subgroup with the highest values of CAP and PWV, had a significantly higher prevalence of metabolic syndrome and its diagnostic criteria.

7. Arterial parameters measured noninvasively were significantly less favorable in those with very high cardiovascular risk according to SCORE2. No patient in the lower SCORE2 risk categories had detectable liver fibrosis by FIB-4 calculation.

8. Lower levels of physical activity were observed in patients with metabolic syndrome, indicating a link between physical inactivity and liver damage associated with this syndrome.

In conclusion, our research has highlighted the complex relationship between metabolic syndrome, liver damage and cardiovascular risk, highlighting the importance of monitoring and managing these issues in patient care.

ELEMENTS OF ORIGINALITY, LIMITS AND FUTURE DIRECTIONS

This thesis aimed to explore the complex relationships between noninvasive liver assessment parameters, atherosclerosis markers and metabolic syndrome. The main novel aspects include:

- Making the case for adopting the new terminology for metabolic dysfunction associated steatotic liver disease (MASLD) instead of the old name (NAFLD).
- Use of simple and accessible methods to assess patients, such as serological scores, followed by TE assessment for those at risk.
- Development of a clustering algorithm to identify subgroups of patients at risk of hepatic as well as cardiovascular disease progression, according to severity of liver steatosis and fibrosis.
- Complex assessment of the correlation between MASLD and cardiovascular risk using noninvasive parameters (CIMT, PWV).
- Highlighting the importance of lifestyle modification and promotion of physical activity in the management of patients with MASLD and metabolic syndrome, given the limitation of pharmacological therapy.

These findings contribute to a deeper understanding of the interactions between MASLD, metabolic syndrome and atherosclerosis, providing insights for more effective and personalized management of these clinical conditions. The noninvasiveaccessible methods used in this study may have a significant impact on patient assessment and management.

Limit

- Absence of invasive methods (liver biopsy, PWV measurement) as a reference for the assessment of liver fibrosis and arterial stiffness, making results obtained with noninvasive techniques not directly verifiable.
- Sample size can be considered a limitation, especially in data analysis using machine learning. However, there are studies that have successfully

implemented CART algorithms to distinguish between dichotomous results in similar domains.

- The presence of confounding factors that may influence outcomes, such as self-reported alcohol consumption or issues related to cardiometabolic criteria, has been minimised but may still influence outcomes.

Future directions

Future research should prioritize the establishment of a unified definition for metabolic syndrome, the adoption of MASLD nomenclature over NAFLD, and the thorough investigation of the relationship between these two entities. This approach will facilitate accurate diagnosisand effective management strategies while maintaining the validity of previous research. Furthermore, gaining a deeper comprehension of the interplay betweenMetS and its effects on various organs is crucial. This involves recognizing metabolic dysfunctions within the spectrum of MetS and devising strategies for lifestyle adjustments to mitigate cardiovascular risk, with a special focus on promoting physical activity.

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ANNEXES

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