“CAROL DAVILA”
UNIVERSITY OF MEDICINE AND PHARMACY
BUCHAREST

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UNIVERSITY OF MEDICINE AND PHARMACY
BUCHAREST

DOCTORAL SCHOOL
MEDICINE FIELD

Contributions to postmortem interval estimation in forensic medicine practice

PhD SUMMARY

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List with published scientific articles (chronological presentation)

1.Web of Science 2026, Original article: **Turlea, IR**; Curca, GC; Mernea, M; Matanie, AC; Fendrihan, S; Mihailescu, DF. FTIR Spectroscopy of Vitreous Humor for Postmortem Interval Estimation: A Multivariate Regression Approach. INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES. 27/8, 3468, Q1 4.9/4,9
LINK poate fi accesat la [httpss://www.mdpi.com/1422-0067/27/8/3468], ref. bibl. [17].

2.PubMed 2026, Case report from the casuistry: **Turlea IR**, Curca GC, Matei L, Stoica A. Death in Epilepsy: Ictogenic Vs. Cardiogenic Mechanisms and Immunoassays in Postmortem Interval Estimation - Case Report. MAEDICA (Bucur). 2026 Mar; 21(1):226-236. doi: 10.26574/maedica.2026.21.1.226. PMID: 41978846; PMCID: PMC13061439,
LINK poate fi accesat la [https://pmc.ncbi.nlm.nih.gov/articles/PMC13061439/], ref. bibl. [22].

3. PubMed 2025, Review: **Turlea IR**, Curca GC. Recent Advances in Forensic Medicine at the Horizon of Omics Sciences and New Biomarkers for Postmortem Interval Estimation. MAEDICA (Bucur). 2025 Dec;20(4):851-863. doi: 10.26574/maedica.2025.20.4.851. PMID: 41537070; PMCID: PMC12767902,
LINK poate fi accesat la [https://pmc.ncbi.nlm.nih.gov/articles/PMC12767902/], ref. bibl. [56]

4.BDI 2025, Review: **Turlea IR,** G. Curca, L. Matei, C. Trandafir, F. Perde. Limitations of ELISA immunological analyses in determining the postmortem interval. Rom J Leg Med33(2)162-165(2025), DOI:10.4323/rjlm.2025.162,
LINK poate fi accesat la [https://rjlm.ro/index.php/arhiv/1052], ref. bibl. [67].

Abbreviation list

ADD, accumulated degree-days, engl.

ATR, Attenuated Total Reflection

CTG, convulsii tonico-clonice generalizate

cTnT, Troponină cardiacă T izoformă cTnT

DAFAB, Departamentul de Anatomie și Fiziologie Animală și Biofizică, Facultatea Biologie, Universitatea București

DPM, data probabilă a morții

ELISA, enzyme-linked immunosorbent assay, engl.

EMSC, Extended Multiplicative Signal Correction

FTIR, Fourier-transform infrared spectroscopy

INML MM, Institutul Național de Medicină Legală Mina Minovici București

PMI, interval postmortem

PMI_C cunoscut

PMI_E estimat

LDA, analiza liniară discriminantă

LOOCV, leave-one-out cross validation

MAE, mean absolute error, engl., eroare medie absolută

OD, ochi drept

OLS, Ordinary Least Squares

OS, ochi stâng

PLS, Partial Least Squares DA, PLS-analiza discriminantă, regresie multivariată

PC, componente principale

PCA, analiza componentei principale

PYTHON limbaj de programare superior cu algoritmi de interpretare

R^2, coefficient de determinare

RAMAN, Spectroscopie

RANSAC, RANdom SAmple Consensus

RANDOM FOREST, algoritmi care îmbunătesc acuratețea predicției și suprapunerile

RMSD, root mean squared deviation, engl. deviația mediei rădăcinii pătrate

RMSE, root mean squared error, engl., eroarea mediei radăcinii pătrate

SERCA, reticulului sarcoendoplasmatic

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, engl.

SVM, Mașini de Suport Vectorial

TSD, Timp scurs de la moarte

UB, Universitatea București

UMFCD Universitatea de Medicină și Farmacie Carol Davila

UV, umoare vitroasă

VIP scoruri cu importanță variabilă în proiecție

Western Blot, immunoblotting, engl.

Introduction

Estimating the postmortem interval (PMI) is one of the important steps in on-site investigations in every death case, having significant legal relevance in cases of suspicious death or homicide, during the investigation or when drawing up the list of suspects. Estimating the PMI presents challenges in judicial investigations and can guide authorities in choosing a lead. In forensic practice, we have encountered cases where determining the PMI later helped uncover the truth. The topic I chose, PMI estimation, is a relatively new factor at the national level, where I conducted two studies on human biological samples, benefiting from the support of interdisciplinary teams and access to specialized proteomics and metabolomics laboratories: the experimental metabolomics study was carried out by an interdisciplinary team of forensic doctor-biologist-physicist and led me to the Department of Anatomy, Animal Physiology, and Biophysics (D.A.F.A.B.) at the Faculty of Biology, University of Bucharest, UB; *the non-experimental proteomics study* to work in an interdisciplinary team as a forensic doctor with a biologist and a laboratory doctor in the Medico-Legal Serology Laboratory at the National Institute of Forensic Medicine "Mina Minovici" Bucharest. The research hypothesis in the metabolomics study was the following: if we select a batch (cohort) of deceased hospital cases with known PMI (post-mortem interval), without any other special selection criteria in order to maintain the heterogeneity and complexity of the cases (stratified randomization), and we collect vitreous humor, VH, which we then examine spectroscopically using the ATR-FTIR technique, we will know the metabolic spectrum of the VH of these cases at different known PMIs, to which we will apply multivariate regression to obtain a corresponding spectroscopic model that we then apply to real cases in the field with an unknown PMI that was estimated using conventional on-site methods, PMI_{Est} will verify the correspondence between the conventional estimate and the ATR-FTIR spectral estimate with the aim of applying this model to cases with unknown PMI in real forensic casework. The research hypothesis in the proteomic study was as follows: if we select a hospital case where the person died at a known time (day, hour, minute) with a known PMI relative to the autopsy time (day, hour, minute), 36.8 hours, and we collect skeletal muscle during the forensic autopsy as a biological sample to determine in the immunology laboratory the autolytic degradation values of 3 protein biomarkers: vinculin, desmin (cellular cytoskeleton proteins), and cardiac Troponin T, cTnC Using the ELISA technique (enzyme-linked immunosorbent assay) and vinculin through SDS-PAGE (sodium

dodecyl sulfate–polyacrylamide gel electrophoresis) with Western Blot (immunoblotting), we could compare the resulting values with the specialized literature, which would allow us, on a selected case series, to assess the usefulness of the protein degradation study for estimating PMI, and on the other hand, we could use the obtained results as reference values, a benchmark, for the studied PMI, 36.8 hours.

Limitations of the research conducted.

Among the limitations of metabolomic study, I can list:

- The relatively small number of cases
- The pseudo-stratified random selection of cases did not differentiate between causes of death, hospital treatment received, or other conditions, so the interference of internal factors (confounding factors) and external factors (extraneous factors) was complex (this heterogeneity of cases as a limitation led the research design to focus on real forensic cases where the medical examiner does not select the causes of death), PMI was an independent variable for the hospital case cohort and a dependent variable for the field case cohort (this limitation led the research design toward real medicolegal cases)
- The PMI calculation for field cases depends on environmental factors like temperature, humidity, presence of clothing, placement on/in the ground, type of soil, etc., as well as the time when the discovery of the body was reported, etc.
- The lack of detailed environmental data (especially temperature, humidity, etc.) has prevented the development of models adjusted for temperature.
- The lack of knowledge of the UV composition through electrophoresis before spectral analysis.

Among the limitations of the proteomic study, I can list:

- Only a single selected and presented case
- The cause of death (epilepsy with grand mal seizures and generalized convulsions) is a limitation since it may interfere with protein degradation patterns due to changes in muscle metabolism (regenerative skeletal muscles with possible rhabdomyolysis) Although PMI is known, 36.8 hours, body cooling temperatures until it reaches 40°C, the refrigeration temperature is not known (ADD calculation [°d] = time [d] × temperature [°C]). All temperatures need to be known and taken into account: ambient temperature where the body is cooling, cooling time, refrigeration

temperature, autopsy room temperature, lab working temperature (even if preparation is done on ice)

- Lack of creatine kinase control for rhabdomyolysis
- ELISA has a complex working protocol that requires good knowledge and specialists

Perspectives for continuing the research. For the most useful application in forensic practice and an increased translational value stemming from interdisciplinarity, the research needs to continue both metabolomically and, especially, proteomically, in order to provide validity, to verify the results, and to confirm the newly identified aspects so that it offers not only exploratory value but also experimentally validated value. The research can continue by keeping the current design of the metabolomic study, and expanding it to the proteomic one as well: two cohorts, one with known PMI, PMIC deceased in the hospital, to build a model that will then be applied to cohort 2, cases from the field. Case selection should consider (1) internal conditions (cause of death, comorbidities, and treatment applied in the hospital), (2) knowledge of temperatures in the field cases (including weather), (3) selected PMI intervals chronologically: PMI 24-26, PMI 36-48, PMI 48-72, PMI 72-96 hours, and > 96 hours. We appreciate that the cohort method is superior to intracorporeal studies at different PMI intervals because the latter would involve prolonged refrigeration of the corpse and repeated sample collection, as well as the extracorporeal method, which involves freezing and thawing samples at different PMIs, creating lab conditions that are very different from real cases, (4) knowing the internal/external conditions (confounding and extraneous factors). Another perspective is aligning PMI in laboratory studies with PMI of body decomposition (for example, a short post-mortem interval, as defined in laboratory studies, < 7 days, is partially included in the early post-mortem decomposition phase and partially in the late post-mortem decomposition phase, while the intermediate post-mortem interval <120 days and the late post-mortem interval (> 120 days) both belong to the late body decomposition phase) with the correlation of PMI conceptualization and body decomposition.

I. General section (actual status of knowledge)

1. PMI estimation using conventional methods

1.1 Cadaver signs

1.1.1. Lividities (*livor mortis*). After the cessation of cardiovascular activity under the action of gravity, fluid blood gradually accumulates in the dependent capillary bed, where it causes a purplish-blue coloration: it starts within 1-2 hours, becomes noticeable from 2-4 hours, and is fully developed at 10-12 hours [1].

1.1.2 Rigidity (*rigor mortis*)

The lack of oxygen after breathing stops initially leads to a decrease in ATP, then to resynthesis through anaerobic glycolysis and the creatine kinase reaction, then to resource depletion with conformational changes in actin and myosin filaments and the formation of bridges between myofilaments. As ATP decreases, the decoupling of actin-myosin (below 85%) in muscle fibers also decreases, leading to rigidity [2,3] that affects all muscles at once but becomes clinically noticeable after 2-4-6 hours in the muscles of the head (eyelids, orbicularis, facial, jaw muscles, smaller muscles) and then progresses according to Nysten's law (1881), the "rigor march" head, trunk, lower limbs, upper limbs, reaching generalization after 12 hours. As the actin-myosin complex undergoes proteolysis [4] and putrefaction begins, rigidity starts to resolve, completely disappearing by around 36 hours [5,6].

1.1.3 Body cooling (*algor mortis*)

Body temperature (measured rectally) is probably the most reliable method for determining early PMI (the 'gold standard') under known and stable external temperature conditions [49]: the drop is about 1°C/hour (1.5 °F/hour) in the first 12 hours and 1.5 °C/hour (1 °F/hour) in the next 12-18 hours, twice as fast in tropical climates [50]. Hennsge's nomogram is the gold standard for early PMI in the first 10 hours according to B = -1.2815 (body weight -0.625) + 0.0284, showing that the equation depends on body weight [7, 8].

1.1.4 Autolysis *(autolysis)*

After death, tissues are affected by their own enzymes, which come into play as cell and intracellular membranes break down, actively breaking down organic structures and various protein, lipid, nucleic, and other molecules and compounds. Autolysis happens without using energy and affects all cells, tissue structures, and body organs. It forms the basis of putrefaction and body decomposition, being the reverse process of building, synthesizing, and organizing

body structures. Autolysis is accompanied by changes in the composition of the whole organism, but in recent years, the most studied changes have been the thanatochemical ones (thanatochemistry studies) [9] of the eyeball, such as those concerning hypoxanthine [10, 11], potassium [11], and metabolites in the vitreous humor (metabolomics studies) by Zhang J. starting in 2018 [12], later benefiting from ATR-FTIR, Fourier transform infrared spectroscopy [13,14,15,16], a scientific interest that also includes my own metabolomics study from the present doctoral research [17]. Thanatochemistry provides analyses of chemical changes in human biological fluids, such as in pericardial fluid [18], cerebrospinal fluid, including in comparative studies, for example with vitreous humor [19], etc.

The first proteomics studies on protein degradation in autolysis were conceived by Pittner S. in 2015-2016 [20,21], a scientific context which also includes the author's own proteomics study from the current doctoral research [22].

1.2 Cadaver decomposition stages and PMI

The decomposition of the body goes through several stages once autolysis begins the process of breaking down the highly organized structure [23]: the early stage (fresh), which includes autolysis and the onset of putrefaction, the initial stage (putrefaction), the progressive stage, and the skeletal stage [5]. Other authors [24] consider five other stages suitable, which can also be used in assessing PMI:descompunere inițială (1-3 zile) echivalent fresh state, initial decay, engl.

a. bloated putrefaction (4-10 days) equivalent to bloat state, putrefaction, Eng.

b. black putrefaction (10-20 days) equivalent to active decay, black putrefaction, Eng.

c. butyric fermentation (20-50 days) equivalent to advanced (post decay), Eng.

d. dry decomposition 50-365 days equivalent to dry decay, Eng.

e. human remains: 365 days (Payne, [23])

Conventional changes are usually accepted in court by investigative authorities or by judges, especially those supported scientifically by lab results, like temperature measurements.

1.4 Forensic entomology

There are two methods for estimating PMI using forensic entomology [25]:

1) assessing insect succession (succession wave), which depends on the geographic area and environmental conditions [26].

2) tracking the development of immature insects collected from the corpse, which are observed in a controlled environment [25].

1.5 PMI estimation at the crime scene; case studies

1.5.1 Integrative methods: score methods

The study of decomposition and the use of decomposition scores, especially for the late postmortem stage, become useful [27-31, 32]. A quantitative method called the total body score, TBS [29], has been developed. By combining TBS with accumulated degree-days, ADD [26], PMI can be estimated more accurately when temperature differences are also considered in the context of decomposition, especially advanced decomposition [33]. Other methods include the hepatic decomposition score, HDS [26,27], the whole-body TADS scoring system in aquatic environments [28], the total decomposition score [30], or the universal formula [31].

2. PMI estimation using methods and techniques of laboratory

2.1 Biomarkers

Discovering new biomarkers is really important not just for the development of science, but also for getting biomarkers that are as specific as possible and show better predictive behavior.

2.2. PMI estimation in laboratory: methods, techniques, equippments

PMI can be studied using multiple methods and laboratory techniques like microscopy, ultraviolet fluorescence, infrared microscopy, SEM electron microscopy, chemiluminescence, radiocarbon testing, biochemistry, infrared spectroscopy including FTIR, Fourier-transform infrared spectroscopy or ATR-FTIR, attenuated total reflectance FTIR, RAMAN spectroscopy, which measures light scattering through material, X-ray energy-dispersive analysis, EDX, micro CT, LC-MS, LC-MS/MS, MALDI-TOF, etc.

2.3 Omics sciences

Omics (from English 'omics') is a term that began to be used starting in the 1990s by borrowing a neo-suffix from the Greek language (oma, Greek) to mean a whole, 'a complete set,' 'a totality of something,' in biology 'a totality of molecules' [56]. Today, omics sciences include genomics (DNA), transcriptomics (RNA), proteomics (proteins), metabolomics (metabolites), lipidomics (lipids) [56].

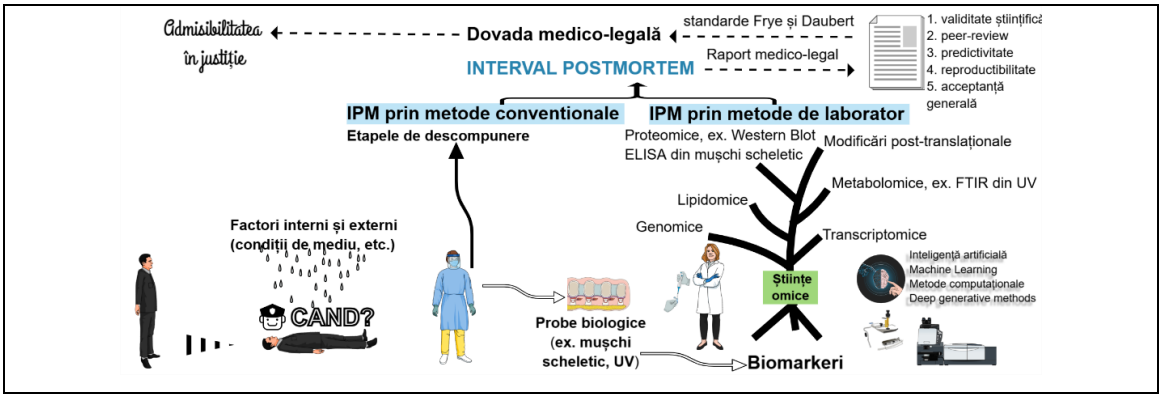


Fig 2 Original graphic representation using Mind the Graph of PMI estimation through conventional and laboratory methods. The burden of proof falls on forensic medicine and forensic experts, who ensure scientific objectivity while adhering to the Frye and Daubert standards and the 5 criteria for admissibility in court: scientific validity and peer review, predictability, reproducibility, and general acceptance. Modified from [56].

2.3.7 Integrative considerations on omics sciences in PMI estimation

To number, just in the year 2025, a total of 22 reviews in this field come to bring knowledge and updates on PMI estimation, which supports an unprecedented development of information in this area, highlighting the importance and relevance of the study topic, PMI estimation, the topic under which the present doctoral study falls, the modernity of PMI research through the development of laboratory methods and techniques, especially in omics sciences regarding PMI estimation [55,56] (research including the metabolomics and proteomics study of the doctoral research that will be presented in the special section), "omics," "multi-omics" in PMI [57], the current understanding of PMI, and future research directions [58]. We expanded on previous research [20,21] regarding human studies that estimate PMI and that use human biological products in the lab, and we managed to identify, updating searches inclusive of 2026, a total of 21 postmortem human studies (Figure 1). Estimating PMI using the methods and techniques of omics sciences as of 2025 highlights 26 studies, of which 17 are proteomics studies, 9 metabolomics studies, 2 lipidomics studies, and 1 multiomics study [34]. Of these, 17 studies focus on early PMI, 8 on mid PMI, and 4 on long PMI [35]. Eleven studies (42.30%) analyzed human tissues and used biological samples from cadavers, while the rest used animal models: 3 studies published results for both animals and humans.

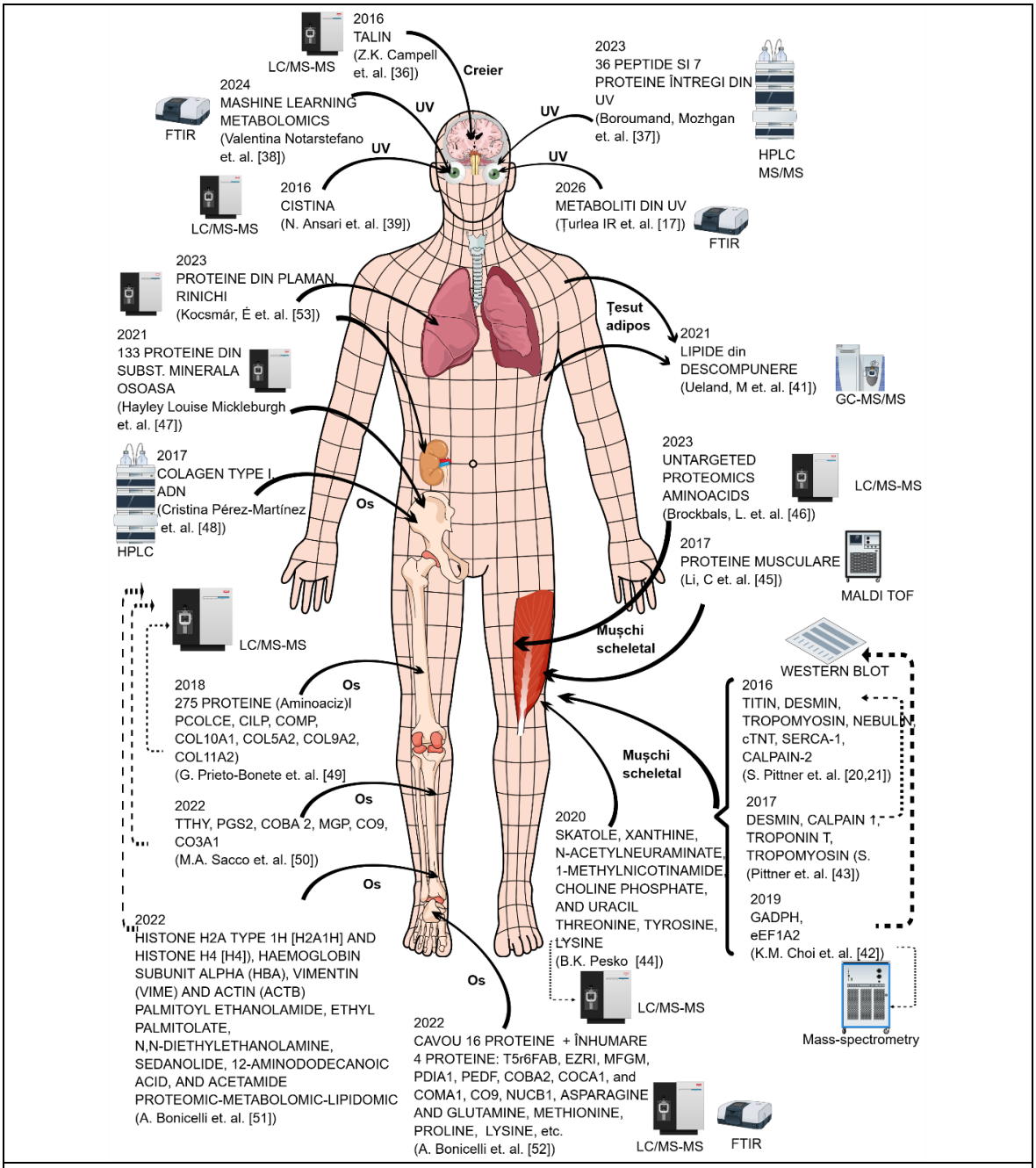


Fig. 1 Infographic representation of research in the field of omics sciences [56]. Our searches led to the identification of 21 human studies, which are shown next to it. Original creation using Mind the Graph, modified after [56].

In our review of the literature, we found 22 human studies, including the present studies [17,22]. Human biological products used include the brain [36], vitreous humor [17,17,38,39], organs (lung and kidney) [40], adipose tissue [41], skeletal muscle (mainly for short PMI estimation) [42,20,21,43,44,45,46], bone and ligaments (mainly for long PMI estimation)

[47,48,49,50,51,52]. Techniques used include LC/MS-MS [36,53,47,49,50,51,52,44], mass spectrometry [42], HPLC and HPLC-MS [37,48], FTIR [35,52], MALDI-TOF [45], Western Blot immunoassay [20,21,43].

2.6.2 Data reproductibility and interpretation of results

Knowing the internal/external conditions and selecting cases that take these factors into account would lead to data with increased reproducibility.

2.6.3 Justice admissibility of PMI estimation data and results

The burden of proof lies with the forensic expert, who validates the data and laboratory results through their conclusions. This involves correlating the data from the perspective of reproducibility, acceptance, and scientific validity—that is, the Frye and Daubert standards for them to be admissible in court, which of course is up to the justice system. Complementarity, interdisciplinarity, transdisciplinarity, and translational research can boost admissibility: the studies conducted were both interdisciplinary and translational.

2.6.4 PMI estimation using laboratory data: ethical aspects

Respecting research ethics, informed consent, ethics committee approval, the Declaration of Helsinki, and the CMR's Medical Code of Ethics.

Original section. Personal contributions

3. Hypothesis and general objectives

The research has *the general aim* of highlighting the usefulness of laboratory investigations in estimating PMI by analyzing postmortem degradation of metabolites (metabolomics study) and postmortem protein degradation (proteomics study). At the scene, judicial authorities always request an estimate of PMI, which is assessed through conventional methods (postmortem changes, stage of livor mortis, rigor mortis, temperature, stage of dehydration, early putrefaction signs, body decomposition, larval activity, etc.) that forensic experts integrate with investigation data collected from witnesses, etc., but which can be refined by narrowing down the PMI and reconfirming the data obtained through conventional methods using laboratory investigation results.

The overall goal is to capture postmortem biochemical and molecular changes in the metabolites and proteins of human tissues and biological products, and to identify predictive behaviors useful for estimating the postmortem interval, especially during the early PMI period. The specific objectives will be detailed along with the presentation of each study. The general working hypotheses were presented in the Introduction chapter.

4. General methodology of the present PhD research

Both research studies were conducted on humans and human biological products: the metabolomics study is an experimental study, while the proteomics study is a non-experimental observational study, in which the degradation of vinculin, desmin, and cardiac troponin T in skeletal muscle was investigated. Unlike the experimental study, the non-experimental study can take into account not only the independent and dependent variables but also other factors such as third-party factors (extraneous factors) and internal factors that may be confounding in research, allowing for a more complete assessment of protein degradation, as happens in a real-life case. The metabolomics study has PMI as the independent variable in the control group (cohort 1), where by selecting cases with a known PMI, PMIC, an attempt was made to develop a possible metabolomic model (from FTIR spectral analyses in UV) which could then be applied to the study group (field cases). There, at the site, with an unknown PMI, an estimated PMI, PMIE, was determined using conventional methods, where PMI is the dependent variable, meaning it is measured (evaluated). Proteomics research, on the other hand, uses PMI as the

independent variable (which is known from the start) in the case studied: the dependent variables (which are measured) are the proteins studied, vinculin, desmin, and cardiac Troponin T in their degradation process in skeletal muscle. The research conducted in the metabolomics study uses two mathematical tools capable of handling large databases and predictively correlating multiple variables: (1) the Fourier series, which underlies the algorithm that allows the analysis of data received by the infrared spectrometer within the FTIR (Fourier Transform Infrared Spectroscopy) equipment, and (2) multivariate regression. In the general methodologies used in the research carried out for both metabolomics and proteomics studies, we can also include adherence to ethical principles.

5. Metabolomic study of cadaver humor vitreous using ATR-FTIR Spectroscopy and multivariate regression

5.1 Introduction. Purpose, hypotheses, specific objectives, aspects already presented in the introduction of the summary.

Specific objectives of the metabolomic study:

a) Extensive review of the specialized literature

b) Spectral analysis of cadaveric UV

c) Developing a protocol for the transport and biosecurity of biological samples

d) Using statistical interpretation methods (multivariate regression, etc.) to generate a spectral model that can be applied to field cases with unknown PMI, estimated PMI, so as to validate PMI through conventional methods and verify the applicability of the model in real cases.

e) Establishing the limits of applicability of the model based on the metabolomic profile of UV

f) Identifying directions for further research

5.2 Material and method

5.2.1 Vitreous humor It was collected from the corpse during the autopsy from both eyes, OD, OS, in each case.

5.2.2 FTIR (Fourier Transform Infrared Spectroscopy), ATR-FTIR (Attenuated total reflection)-FTIR. It was used the spectroscopy as a method with infrared beam as a technique

using as instrument ATR-FTIR for infrared spectral analysis of the matolites remained in the VH after deproteinization.

5.2.3 Place where the research was performed, temperatures. It involved both INML Mina Minovici Bucharest (forensic autopsy, sample collection, initiating the transport protocol) and the Department of Animal Anatomy and Physiology at UB, DAFAUB (sample storage, processing, spectral analysis, statistical interpretation through multivariate regression).

5.2.4. **Case selection:** Cohort 1, 20 in-hospital deaths (hospital cases), with known PMI, PMIC (control group), inclusion criteria: in-hospital deaths with known PMI, PMIC (stratified randomization), exclusion criteria: non-hospitalized cases; and Cohort 2, 10 on-site deaths, PMIE (study group) with inclusion criteria: non-hospitalized deaths with unknown PMI, where PMI was estimated using conventional PMIE methods, exclusion criteria: in-hospital deaths (admitted cases) or with known PMI.

5.2.5. PMI calculation. PMI calculation for cohort 1 and cohort 2

PMIC = (Date of the autopsy at the time of the autopsy, day, hour, minute) – (Date of death at the time of death, day, hour, minute), known PMIC date or estimated PMI_{EST}

5.2.6 Casuistry

5.2.6.1. Cohort 1 (hospital cases) with known PMI, PMIKn [17].

5.2.6.2. Cohort 2 (crime scene cases) with unknown PMI, estimated using conventional methods, PMIEst [17].

5.2.7 Sampling protocol for vitreous humor. In both eyes, OD, OS, 1ml of UV on ice each.

5.2.8. Biosecurity protocol for cadaver sampling: good laboratory practice. A biosecurity protocol was developed (which includes sample collection, storage at INML MM, transport, storage at DAFAB UB, sample processing using ATR-FTIR in the DAFAB UB lab, spectral analysis, data interpretation, and returning biological waste to INML MM) [17].

5.2.10 FTIR data analyze methods. The data analysis was done with Python (v3.11.7; Python Software Foundation, Wilmington, DE, USA) using the libraries NumPy (version 1.21.0), pandas (version 1.3.0), SciPy (version 1.7.0), and scikit-learn (version 1.0.2) [17]. It included Spectral Processing, Second Derivative Transformation with Mean Centering, Peak Intensity Analysis, Principal Component Analysis (PCA), Partial Least Squares (PLS) Regression, Final Model Calibration, Spectral Interpretation, Binary Sample Classification,

Comparative Analysis of Regression Methods using Ordinary Least Squares (OLS), Partial Least Squares (PLS), Huber Regression, Theil–Sen Regression, and RANSAC (RANdom SAmple Consensus). All regression methods were applied to the same preprocessed spectral data and evaluated using LOOCV. Model performance was assessed using RMSE, MAE, and R2 [17].

5.3. Results

5.3.1 Spectral modification in PMI. FTIR spectra of UV samples with known PMI (cohort 1) were analyzed to characterize the variation of absorption peaks depending on PMI. Examples of FTIR spectra measured on samples with different PMI (range 1800 – 700 cm-1) are shown in Figure 3, [17]. These peaks reflect the main biochemical components of the analyzed UV, including peptides (~1663 cm-1, ~1630 cm-1, ~1315 cm-1), free amino acids (~1414 cm-1), lipids (~1456 cm-1), lactate (~1121 cm-1, ~855 cm-1), glucose and other monosaccharides (~1041 cm-1), nucleic acids (~925 cm-1), or urea, creatinine, and uric acid (~1580 cm-1) [17].

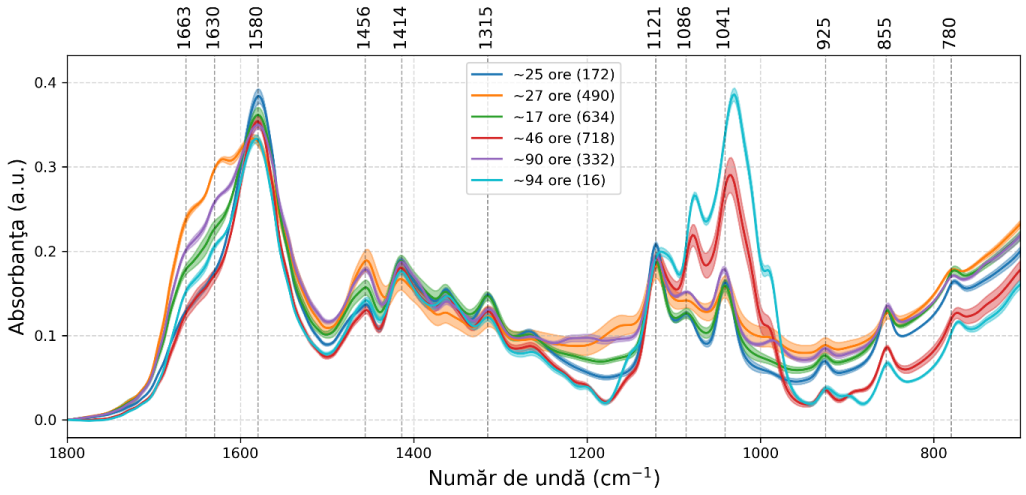


Fig. 3 The FTIR spectra of samples with known PMI: PMIC: 172 (~25 hours, blue line), 490 (~27 hours, orange line), 718 (~46 hours, red line), 332 (~90 hours, purple line), 16 (~94 hours, cyan line), and a sample with unknown PMI: 634 (estimated PMI ~17 hours, green line). The lines represent the mean values obtained from repetitions, and the shades show the standard deviations, n = 4. Diagnostic peaks are marked with dotted lines, and the corresponding wavenumbers are indicated above them [17].

5.3.2. FTIR spectral cases: Case clusters 1-2

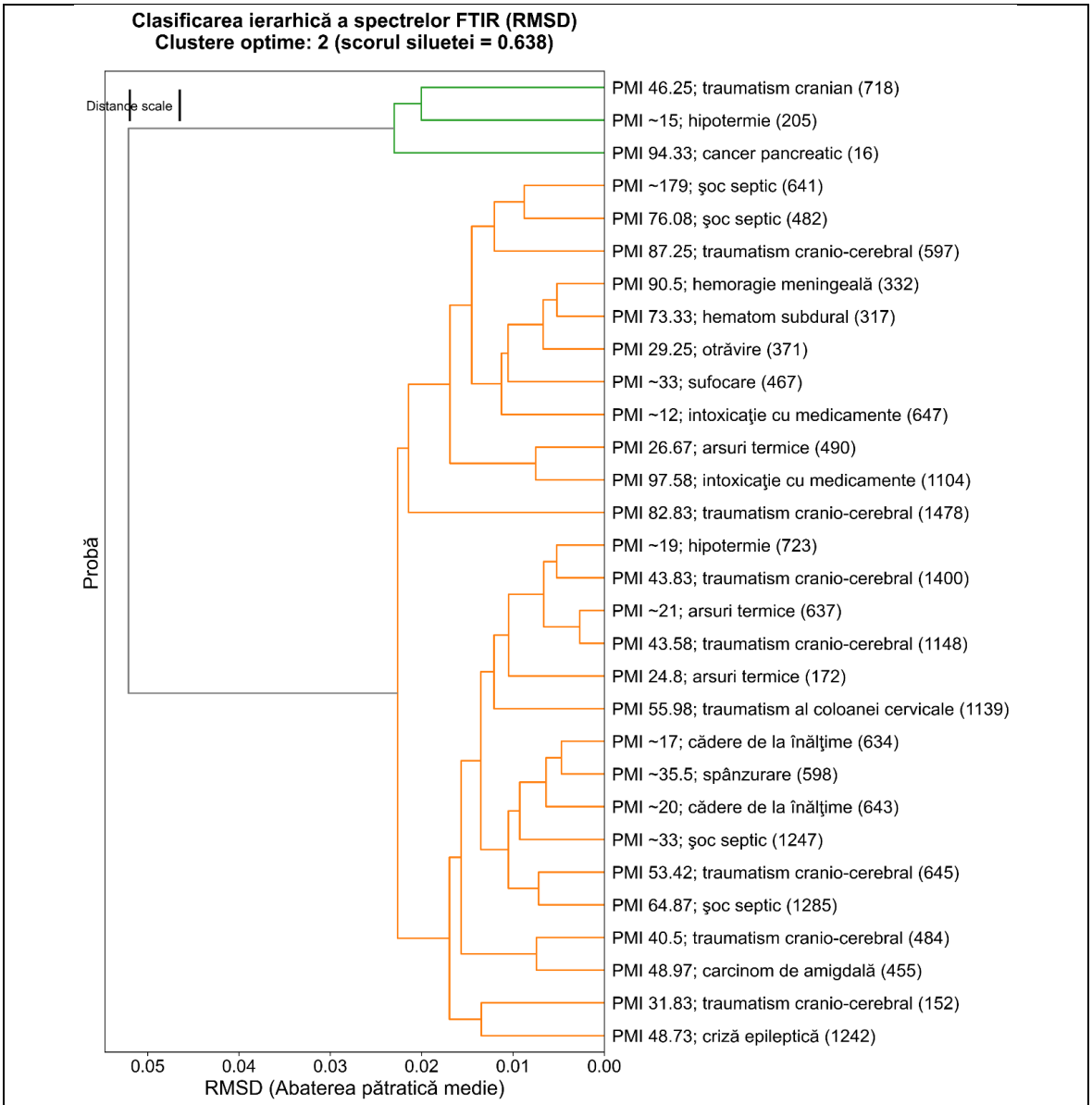


Figura 4. Horizontal dendrogram showing the relationship between the 30 vitreous humor samples. The labels indicate PMI, cause of death, and the sample ID. The optimal solution with two clusters (silhouette score = 0.636) is highlighted with color shades: Cluster 1 (blue, n = 27) and Cluster 2 (orange, n = 3) [17].

The cases were organized into clusters, cluster 1 and cluster 2 (Figure 4) [17]. Cluster 1 includes samples with different case profiles, showing that the spectra are sensitive to the biochemical conditions specific to different traumatic pathologies. Cluster 2 includes three samples: case 16 (PMI 94.3 hours, pancreatic cancer, acute stroke), case 205 (PMI ~15 hours, hypothermia), and

case 718 (PMI 46.2 hours, head trauma and surgery for subdural hematoma). In samples 16 and 718, it is shown that intracranial pathology (stroke and head trauma, respectively) promotes the disruption of the blood-eye barrier, allowing blood-derived proteins, inflammatory mediators, and cell debris to enter the vitreous humor, a point confirmed by studies in the literature [59,60,61]. It has also been shown that hyperthermia can affect the UV composition [17].

5.3.3 Modelling data with regression methods

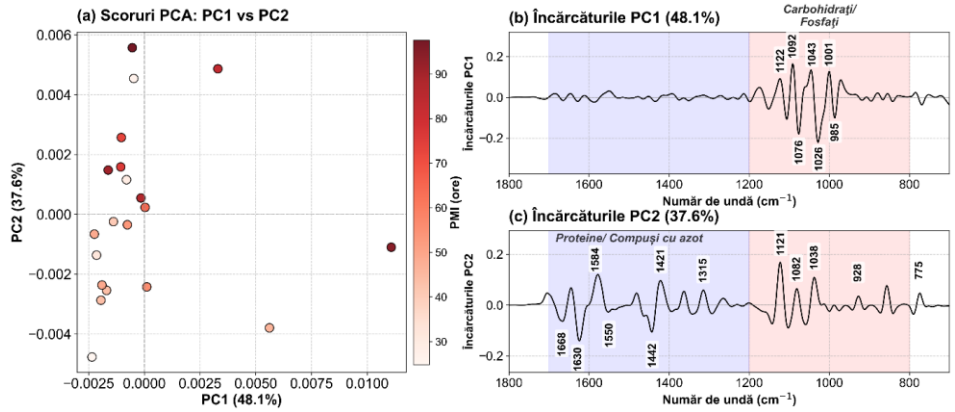


Figura 5. The results of the PCA analysis performed on the VH sample spectra with known PMI, considering the range 1800–700 cm^{-1}. (a) PC1 vs. PC2 score plot. PMI is color-coded. Outlier samples are marked. (b) PC1 loading spectrum. Red was used to highlight the spectral region associated with carbohydrates/phosphates. (c) PC2 loading spectrum. Blue was used to mark the spectral region associated with proteins, and red was used as described in (b). In (b,c) I labeled the important features on the graphs [17].

5.3.3.1 **Principal components of spectral analyze**

Principal component analysis (PCA) was applied to the mean-centered second derivative spectra (1800–700 cm^{-1}) for all 20 UV samples with known PMI. The first two principal components (PCs) explained 85.7% of the total spectral variance (PC1: 48.1%, PC2: 37.6%), suggesting that these two components capture most of the biochemical variation between samples (Figure 5a). The PC1 loading plot (Figure 5b) showed that the most prominent features are concentrated in the 950–1100 cm^{-1} region, which is mainly associated with vibrational modes of carbohydrates and phosphate-containing compounds. Strong positive loadings are observed at 1092, 1043, and 1001 cm^{-1}, characteristic of glycogen (1043 cm^{-1}) [58,59], phosphate stretching vibrations (1086–1092 cm^{-1}), and ribose-phosphate backbone vibrations

(1001 cm^{-1}), suggesting that these biochemical regions represent the largest source of spectral variation between samples.

5.3.2.2 Partial least square, PLS for PMI prediction.

To validate the PLS model, we performed five iterations in which the known PMI spectra were randomly split into a training set (15 samples) and a test set (5 samples). The results show an average test RMSE of 17.9 ± 7.1 h (range 6.7–28.8 h), reflecting the heterogeneity of the dataset and that model performance is strongly influenced by the inclusion of difficult cases, while also showing that the 2-component model (the most common choice) provides a parsimonious and robust solution. The model achieved a cross-validated RMSE of 15.82 h, an R^2 of 0.531, and a mean absolute error of 12.27 h. Based on the absolute prediction errors from leave-one-out cross-validation (LOOCV) for all 20 known samples using the final 2-component PLS model (RMSE = 15.8 h), the samples were divided into three reliability groups: (1) Reliable samples (error < RMSE, n = 14): Predictions within 15.8 h of the true PMI (2) Marginal samples (RMSE ≤ error < 2×RMSE, n = 4): Moderate deviations (15.8 – 31.6 h) (3) Problematic samples (error ≥ 2×RMSE, n = 2) [17]. Interpreting the PLS model through standardized regression coefficients shows the direction and size of the association between each spectral region and PMI: positive coefficients indicate bands that increase in intensity as PMI progresses, while negative coefficients indicate bands that decrease the estimated PMI, error 0.1 h; the most significant positive coefficients were observed in the 1120–1126 cm−1 region, peaking at 1122.5 cm−1 (coefficient = 5.6 × 10−5) [17]. This region is attributed to glycoproteins, small carbohydrates, and phosphodiesters, and it indicates progressive accumulation or structural rearrangement of glycoprotein components depending on the PMI. Strong negative coefficients were observed in the 1070–1080 cm−1 region, especially at 1076 cm−1 (−4.4 × 10−5) and 1074 cm−1 (−4.3 × 10−5). This region is associated with phosphate (PO2−) stretching vibrations and C–O stretching of carbohydrates [63]. The negative sign suggests a decrease in these components over time, in line with the progressive degradation of the membrane. VIP scores (variable importance in projection) used in PLS regression were observed at 1190 cm−1 (VIP = 2.16) and 1192 cm−1 (VIP = 2.13), confirming the dominant role of glycoprotein, small carbohydrates, and the phosphodiester region in predicting PMI. The phospholipid region at 735–739 cm−1 was the next most important (VIP = 2.02–2.10), highlighting the significance of membrane degradation processes [17].

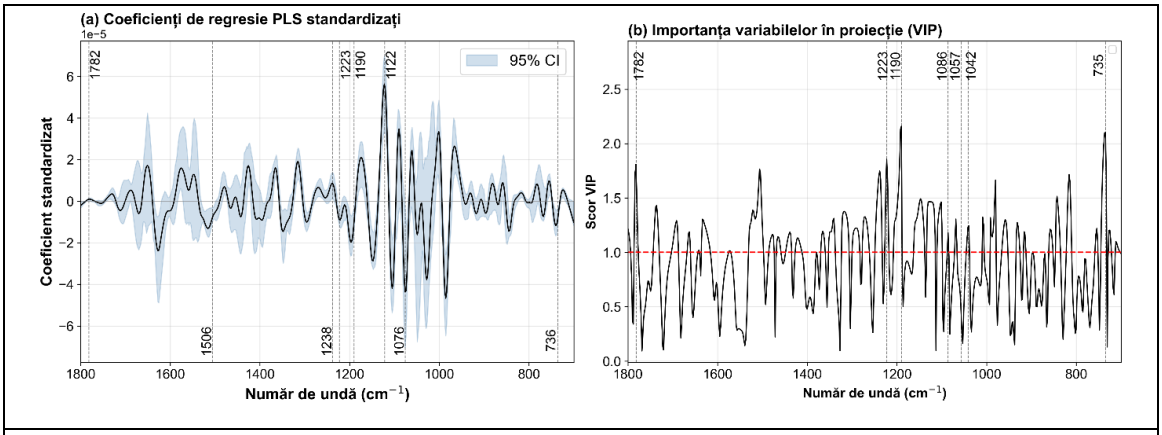


Fig. 6 Interpretation of the PLS regression model (2 components). (a) Standardized regression coefficients for the final model. Shaded areas represent 95% bootstrap confidence intervals. Key spectral regions are noted with approximate wavenumbers. (b) Variable Importance in Projection (VIP) scores. The horizontal red dotted line indicates the VIP threshold of 1.0; wavenumbers above this line are considered significant predictors. Selected VIP peaks are labeled [17].

5.3.2.3 PMI prediction for PMI_{Est} using PLS

The final two-component PLS model, calibrated on all 20 known samples, was applied to the ten samples with unknown or uncertain PMI. The predictions ranged from 27.1 to 80.1 hours. Four samples were classified as outliers, with estimates exceeding the 90% confidence interval: 634 (~17 h, estimated 37.7 h; ~1 h below the lower limit), 637 (~21 h, estimated 48.5 h; ~9 h below the lower limit), 643 (~20 h, estimated 54.6 h; ~14 h below the lower limit), and 205 (~15 h, estimated 67.0 h; ~30 h below the lower limit). Despite the differences in PMI, these samples share similar spectral characteristics.

A notable exception was sample 641 (estimated at ~179 h, planned at 80.1 h; about 79 h over the upper limit of the 90% confidence interval). FTIR spectroscopy captured the biochemical signature of this sample, which seems decoupled from the chronological PMI. In the case of 641, FTIR spectroscopy detects the biochemical age more than the chronological time itself, due to the longer storage period at low temperature, a point also mentioned in other studies [63]. This distinction could have important implications for forensic investigations where temperature history may be unknown or variable [17].

5.3.2.4 Binar classification of samples for forensic triage

We evaluated several classification methods to differentiate between early PMI (≤48 h) and late PMI (>48 h), such as Linear Discriminant Analysis (LDA), Quadratic Discriminant

Analysis (QDA), Support Vector Machines (SVM) with linear and radial basis function (RBF) kernels, and Random Forest. The model's performance was assessed using LOOCV.Rezultatele shows that Random Forest is the classifier that achieved the highest cross-validated accuracy of 70.0% (14 out of 20 samples classified correctly),

Probă	Predicția clasei PMI (precoce, tardiv)	Confidență	PMI_E	Utilitate
647	Precoce (≤48h)	0.59	~12 h	DA
205	Short (≤48h)	0.52	~15 h	YES
634	Short (≤48h)	0.64	~17 h	YES
723	Short (≤48h)	0.83	~19 h	YES
643	Late (>48h)	0.52	~20 h	NO
637	Short (≤48h)	0.64	~21 h	NO
1247	Short (≤48h)	0.64	~33 h	YES
467	Short (≤48h)	0.62	~33 h	YES
598	Short (≤48h)	0.64	~35.5 h	YES
641	Late (>48h)	0.87	~179 h	NO

5.3.2.5 Regression methods comparison in PMI estimation and prediction

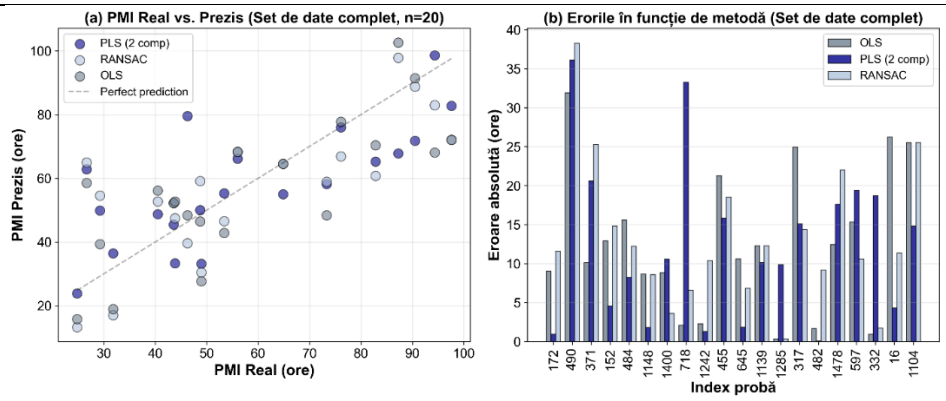


Fig. 7 Comparison of regression methods for PMI prediction. (a) Actual PMI versus estimated (predicted) PMI for PLS, OLS, and RANSAC. (b) Absolute errors on samples for PLS, OLS, and RANSAC, which can be used by multivariate methods like PLS regression [17].

5.4 Discussions

5.4.2 Comparing PhD research values and results with reference values from scientific literature

Previous studies conducted on skin samples taken from humans [37,38,39] and rabbits [12] have shown that ATR-FTIR measurements, supported by chemometric analysis and machine learning, can be used to predict a sample's PMI. The study by Zhang et al. [12] provides a proof of concept for estimating PMI using the ATR-FTIR method under strictly controlled experimental conditions, which we applied in our research [17]. We confirmed the contribution of the 1315 cm^{-1} region (Amide III) to PMI prediction (VIP = 1.37, positive regression coefficient = 1.92×10^{-5}), which represents important cross-species validation, but also found differences in the 925 cm^{-1} band (nucleic acids), which showed a strong correlation in rabbits [12] and minimal contribution in our PLS model for humans (VIP = 1.01, coefficient close to zero, 1.10×10^{-6}), highlighting species differences [17]. In the case of human UV, Valentina Notarstefano and colleagues [38] established a methodology and reported a classification accuracy of over 80% using PLS-DA. They identified discriminant peaks, including those at 1120, 1630, and 1585 cm^{-1}, which can be attributed to protein degradation, amino acid breakdown, lactate accumulation, and changes in hyaluronic acid.

Our results independently validate some of these observations: the glycoprotein/phosphodiester band at 1121 cm^{-1} emerged as the dominant predictor in our PLS model (VIP = 1.47, the highest regression coefficient), while the protein-related bands at 1630 cm^{-1} (β-sheet) and 1580 cm^{-1} (Amide II) showed consistent behavior and led us to hypothesize that they could be robust biomarkers, with a reasonable degree of uncertainty [17]. The band at 1121 cm−1 corresponds to the peak at 1120 cm−1 that we identified. In our VIP-based data analysis, it also identified the phospholipid region at 734–739 cm−1 (VIP = 2.02–2.10) and the lipid ester region at 1782–1784 cm−1 (VIP = 1.72–1.81) as very influential predictors, highlighting the importance of membrane degradation and lipid oxidation, processes that had not been previously highlighted in human VH studies, thus supporting the robustness of these spectral features as PMI biomarkers. Our study builds on the cited reference studies but with more practical applicability because we adopted a design oriented towards forensic practice, which includes cohort 1 with known PMI and a cohort with unknown PMI (from the field) estimated using conventional methods, PMI_{Es}. This way, we addressed the gap between

controlled laboratory conditions and real-world forensic cases. While including cases with PMIE introduces a degree of uncertainty, it provides a more realistic assessment of both the model's advantages and its limitations. We appreciate that what seems like a methodological weakness actually highlights the value of ATR-FTIR spectroscopy, which can support or validate PMI_{Est} on-site using conventional methods but cannot yet replace it [17].

Following the detailed analysis of the aberrant values always correlated with the complexity of the cases (490 – thermal burns, 718 – head injuries), PLS efficiently addresses this by highlighting the robustness of the method against biological heterogeneity. Additionally, the observation that a refrigerated sample (641) was estimated by the metabolomic model at 80.5 hours, even though the conventional PMI is ~179 hours, shows that FTIR detects biochemical age rather than chronological time, a distinction with important implications for forensic cases where the temperature history is unknown [17]. In this way, our study not only tackles the algorithmic performance of PMI prediction models but also allows for a biological and forensic interpretation of spectral changes [17]. It should be noted that our model was calibrated on samples with a PMI ranging from 24.8 to 97.6 hours (roughly 1 to 4 days), corresponding to early to intermediate postmortem intervals. The metabolomic model we present is therefore mainly intended for use within this 24.8 - 97.6 hour range. The applicability of the metabolomic model we identified for shorter or longer PMI intervals still needs to be validated. Environmental factors, like temperature, decomposition conditions, etc., can influence the spectral signatures, as seen in the case of the extended refrigerated sample (641), which showed an estimated PMI of 80.1 hours, even though the known PMI was 179 hours [17].

6. Proteomic study on degrading proteins during autolysis in scheletal muscle to estimate PMI: immunological methods with ELISA and SDS-PAGE with Western Blot techniques

6.1 Introduction (specific scope, hypothesis, specific objectives)

Specific objectives of the proteomics study:

a) Extensive review of the specialized literature

b) ELISA and SDS_PAGE analyses with Western Blot to determine protein degradation for cardiac Troponin T, cTnT, vinculin, desmin with predictive value in estimating PMI

c) Development of a protocol for the transport and biosecurity of biological samples

d) Comparing immunoassay data with similar results from the specialized literature to assess the predictive value of protein degradation for estimating PMI, especially early PMI.

e) Using biomarker values as benchmark values for future studies

f) Establishing the limits of using the results obtained in other real cases

g) Identifying directions for further research

6.2 Material and method

6.2.1 Enzyme immunoassays ELISA, Electrophoresis SDS-PAGE for protein separation and Western Blot for specific protein detection

6.2.1.1 ELISA (enzyme-linked immunosorbent assay). A semi-automated ELISA was applied, in which an antigen is captured through a free epitope by two antibodies, the capture antibody and the enzyme-labeled detection antibody ELK Bio-Rad, using specific software (Magellan).

6.2.1.2 SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with Western Blot. SDS-PAGE with the Pierce BCA testing kit from Thermo Scientific for protein identification by Western Blot and protein identification by Western Blot.

6.2.2 Vinculine, desmine, cardiac Troponine T, cTnT in scheletal muscle. The biological product analyzed was cadaveric skeletal muscle (quadriceps muscle). The biomarkers we looked for were cardiac Troponin T, cTNT, vinculin, and desmin through ELISA, and vinculin for SDS-PAGE with Western Blot. The most commonly used study protocol (extraction, protein identification) is provided by S. Pittner [20,21,43,64] in extracorporeal research, a design that we also adopted and used ourselves.

6.2.3 Research type, sampling

For the proteomics study, a non-experimental observational research was carried out in which PMI was the independent variable, in relation to which the degradation of cytoskeletal proteins desmin, vinculin, and cardiac troponin T in skeletal muscle was investigated to estimate PMI through autolytic protein degradation analysis using immunological methods and ELISA techniques for desmin, vinculin, and cardiac troponin T, and SDS-PAGE with Western Blot for vinculin. Human skeletal muscle was chosen because it can be collected right at the scene and has a slower rate of autolysis in the early postmortem stage. Vinculin was chosen because it is less commonly studied in humans.

6.2.4 Casuistry and limits of case selection

One case was investigated, chosen from the cases of cohort 1, in-hospital deaths, with PMIC, specifically case 1242, which was also the subject of the Pubmed scientific publication [22].

PMI was calculated after the formula [22]:

PMIC = [date and time of autopsy (24.10.2024, 9:30)] – [date and time of death (22.10.2024, 20:46)] = 36.8 hours

6.2.5 Biosecurity protocol

The same biosecurity protocol was applied as in the metabolomic study, which for the proteomic study included collecting biological samples from skeletal muscle, transporting them to the serology laboratory at INML MM, storing them, processing samples through ELISA, SDS-PAGE, and Western Blot, interpreting the results, and destroying the biological material used.

6.3 Results

6.3.1 Value results and comparison with reference scientific studies in literature

6.3.1.1 ELISA results: value and limits in PMI estimation. ELISA provided quantitative values with high sensitivity for cTnT 178.85 pg/mL, vinculin 570.96 pg/mL, and desmin 565.77 pg/mL in human skeletal muscle at a PMI of 36.8 hours [22], which can be used as reference values for similar cases in future studies. The result is notable because other studies have failed to detect cardiac Troponin T in skeletal muscle using ELISA: our favorable result is probably also due to the fact that in the case studied, where the person died during a tonic-clonic

seizure with a diagnosis of epilepsy, the skeletal muscle has regenerative characteristics due to a history of repeated convulsions [22]. I found only one reproducible study in the literature with significant similarities to our study for comparison, Bodor, GC, 1997 [66]: the ELISA method was used in both studies, a similar PMI range (30-40 hours), sample collection from skeletal muscle, muscle with regenerative conditions (epilepsy with rhabdomyolysis due to generalized tonic-clonic seizures, GTCS vs. polymyositis and Duchenne muscular dystrophy [70]). Among the notable advantages of the ELISA technique is its ability to produce accurate values for the analytes studied, which can serve as reference points or benchmark values [67]. The limitations of ELISA immunoassays are mainly due to the complexity of the technique, the need for specialists, the accuracy of standard curves, and so on [67]. 6.3.1.2 SDS-PAGE with Western Blot: value and limits in PMI estimation. SDS-PAGE electrophoresis with Western Blot confirmed the detection of the native 117 kDa band usually seen in reference studies with human samples in early PMI < 0.5 days [65], <24 hours [68], or on postmortem days 1, 2, 3 [69], as well as its degradation products through autolysis corresponding to the 84 kDa bands [64] usually seen on day 1 (24 hours) and 90 kDa bands usually seen on day 3 postmortem [70]; thus, both the method and technique used, as well as the known hospital PMI of 36.8 hours, were validated. Additionally, we managed to identify a degradation product that other studies hadn't spotted [42,21,64], corresponding to the 87 kDa band, which could also serve as a benchmark value for a PMI of 36.8 hours. In the case we studied, we weren't able to identify the 75 kDa and 63 kDa bands [65] that are usually present in intermediate PMI (24–72 hours), but this doesn't diminish the success of determining protein degradations or the prospects for continuing this research, since we demonstrated, by comparing with similar results from international reference studies, the usefulness of using protein degradation in skeletal muscle to estimate PMI [4]. Other human case studies show vinculin degradation bands at 90 kDa and 84 kDa for intermediate PMI < 72 hours [71], which therefore also validates our findings for intermediate PMI and in the specific case with a PMIC of 36.8 hours.

6.4 Discussions

6.4.1 Prediction value in PMI estimating and limits using degradation of vinculine, desmin and cardiac Troponin T in scheletal muscle; limits. The proteomics study confirms the value of using protein degradation to estimate PMI, particularly vinculin, desmin, and cardiac Troponin T. The limitations have already been presented [67].

7. Conclusions and personal contributions

7.1. Conclusions of both studies

The metabolomics study sets a realistic benchmark for what ATR-FTIR spectroscopy can offer in real forensic scenarios and positions ATR-FTIR spectroscopy as a complementary tool to conventional forensic estimation that can strengthen PMI assessments in forensic practice.

a) The research conducted in the metabolomics study showed the following [17]:

- The two-cohort model (cohort 1, known PMI PMIC from the hospital, and cohort 2, estimated PMI PMIE on-site and PMIE via ATR-FTIR with results comparison) showed robust performance on heterogeneous samples, reaching a cross-validated RMSE of 15.82 hours on the full dataset and providing reliable predictions for the ten samples with on-site PMIE.

a) Multivariate PLS regression with two components is capable of extracting predictive information from complex samples, with confounding clinical factors that simpler linear methods cannot utilize, and maintaining stable performance (RMSE = 12.44, R2 = 0.075, MAE = 10.01 h), which in the presence of complex clinical cases has shown the failure of other methods (OLS and RANSAC), and supports its suitability for forensic applications.

b) The Random Forest binary classification model provides a practical screening tool with 70% accuracy, outperforming LDA (60%) and offering confidence scores above 0.9 for 70% of unknown samples.

c) The carbohydrate/glycoprotein region (1190-1192 cm^{-1}) as well as the membrane phospholipid region (734-739 cm^{-1}) as new biomarkers in PMI estimation, which can be which can be more robust than some traditional protein bands

d) The observation that long-term storage in the fridge (sample 641) separates biochemical age from chronological age suggests the need to develop temperature-corrected models, which will further increase their usefulness in the forensic field

e) The practical advantages of the method for determining the metabolic composition of UV by ATR-FTIR are: (1) practical applicability, (2) minimal sample preparation, (3) fast results, (4) transferable through interdisciplinarity to the forensic field (translational value).

b) Studiul de proteomică centrat pe demonstrarea degradării proteinelor vinculina, desmina și Troponina T cardiacă în mușchiul scheletic uman, a identificării valorii și limitelor imunotestelor ELISA, SDS-PAGE cu Western Blot în estimarea PMI, a confirmării rezultatelor obținute prin comparare cu referințele științifice în domeniu și a dezvoltării unor noi date a relevat [22]:

a) ELISA can accurately detect the analytical values for all three selected biomarkers in skeletal muscle (cardiac Troponin T, cTnT 178.85 pg/ml, vinculin 570.96 pg/ml, and desmin 565.77 pg/ml), and these values can serve as benchmark values for a known 36.8-hour PMI.

b) The SDS-PAGE and Western blott results for vinculin degradation in human skeletal muscle are validated by international studies, showing that at an intermediate PMI (PMI of 36.8 hours), the native vinculin band is absent and degradation bands appear at 90 kDa and 84 kDa.

c) Additionally, using SDS-PAGE and Western blott, we were able to identify a degradation band at 87 kDa, which other studies have not reported; this finding needs to be verified and could also serve as a benchmark value for an intermediate PMI (36.8 hours).

7.2 Personal contributions in both studies

The research carried out in the two studies, on metabolomics and proteomics, generated the following personal contributions (side-by-side presentation) [17,22]:

The metabolomics study	The proteomics study
This study analyzed the spectral composition of the vitreous humor, UV using the ATR-FTIR technique to estimate PMI (interdisciplinary and interinstitutional study)	This study analyzed the degradation of the proteins vinculin, desmin, cardiac Troponin T in skeletal muscle using ELISA, SDS-PAGE, Western Blot techniques to estimate PMI (interdisciplinary study))
Both studies, are studies on human biological products (one of the 21 human studies in the literature regarding PMI estimation) which allows for direct practical use of the results in forensic practice with probative value in court	

<table>
<tr><td colspan="2">Build-up a biosafety protocol for using human biological samples in a good clinical practice laboratory applicable to both studies (collection-transport-storage-processing-analysis)</td></tr>
<tr><td colspan="2">Both studies has translational value (transferable through interdisciplinarity to the forensic field with increased evidentiary value in court)</td></tr>
<tr><td>The first metabolomics study on human fingernails published nationally with IF > 4 about estimating PMI by analyzing the spectral composition of human nails
The 12th study on humans regarding PMI
The 4th study on humans regarding PMI estimation in fingernails, from our knowledge the only one on humans regarding PMI estimation in fingernails with ATR-FTIR
The first national study with ATR-FTIR on human fingernails.</td><td>The first proteomics study published in PubMed in the country on estimating PMI through protein degradation in human skeletal muscle</td></tr>
<tr><td>The model structured on two cohorts (PMIC and PMIE), an original study design, using two-component PLS multivariate regression, is robust in its predictions of unknown PMI, especially when there is increased heterogeneity and complexity of cases (PMIE on site)</td><td>ELISA values for cTnT 178.85 pg/ml, vinculin 570.96 pg/ml, and desmin 565.77 pg/ml at 36.8 hours PMI with benchmark value for the study PMI.</td></tr>
<tr><td>Multivariate regression and binary Random Forest classification on the imagined model have the best and most solid results.</td><td rowspan="2">87 kDa vinculin degradation band by Western Blot that could be specific for PMI at 36.8 hours, with a benchmark value for the study PMI range (validation needed),</td></tr>
<tr><td>Carbohydrates/glycoproteins and phospholipids released by membrane rupture turn out to be new biomarkers</td></tr>
</table>

with strong predictability and superior predictive signals and stability in estimating PMI	
Prolonged exposure to cold disconnects the time-dependent biochemical changes in the vitreous humor (especially in carbohydrate metabolism) and highlights the importance of knowing and controlling temperature when estimating PMI (including weather history).	
The practical advantages of the method for determining the metabolic composition of UVs by ATR-FTIR and using multivariate regression in data analysis are: (1) practical applicability, (2) minimal sample preparation, (3) quick results, (4) robustness in statistical interpretation through multivariate regression suitable for increased heterogeneity and complexity of cases, (5) practical applicability and transferable translational value through interdisciplinarity in PMI estimation.	

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