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Bloodstain Proteomics as a Molecular Clock for Estimating Time Since Deposition, Biological Age, and Sex in Forensic Investigations

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ABSTRACT

Blood is one of the most informative biological traces recovered from crime scenes. Beyond conventional DNA profiling, its proteome may provide contextual information concerning when a stain was deposited and broad biological characteristics of the donor. This critical review examines three interconnected applications of forensic blood proteomics: time since deposition estimation, biological-age estimation and biological-sex estimation. We evaluate evidence from haemoglobin oxidation, plasma ageing signatures and sex-associated proteins; examine environmental, substrate and donor-related confounders; and compare the statistical and machine-learning approaches used for prediction. Six proteins, ITIH3, HSPA9, SNAP91, FTL, XPO4 and NCF2, currently provide the strongest replicated feature-selection evidence for age association in dried bloodstains. We further propose a nanoLC-timsTOF diaPASEF workflow for future validation, combining trapped-ion mobility, rigorous quality control, and multi-output models to simultaneously estimate several forensic attributes. Although a unified assay has not yet been validated in a single representative bloodstain cohort, the available evidence supports further development of proteomics as a complement to DNA profiling for crime-scene reconstruction.

1. Introduction

Blood is the most frequently encountered biological trace at violent crime scenes and represents one of the most information-rich substrates available to forensic investigators [1,2]. Traditionally, bloodstain analysis has served two primary purposes: confirming that the substance is human blood and identifying its donor through DNA short tandem repeat (STR) profiling. Although conventional DNA profiling primarily addresses individualisation and can provide sex-associated information when sufficient DNA is recovered, it generally provides little or no information about time since deposition (TSD) or donor age, and it may fail when traces are highly degraded or environmentally compromised [3,4]. These gaps have motivated growing interest in complementary molecular approaches. RNA-based methods have been explored for body-fluid identification and for TSD estimation by exploiting measurable changes in transcript abundance and integrity in dried stains [5,6]. Spectroscopic methods, particularly Raman, FTIR, and UV-Vis spectroscopy, monitor the characteristic colour change from oxyhaemoglobin to methaemoglobin and haemichrome as a proxy for elapsed time [7-9]. Metabolomics approaches using high-resolution liquid chromatography-mass spectrometry (LC-HR-MS/MS) have identified circadian and time-varying metabolites in bloodstains [10,11]. However, none of these approaches has achieved widespread implementation in routine forensic practice because of persistent challenges involving reproducibility across diverse conditions, limited standardisation and validation, insufficient reference datasets, and the absence of integrated models addressing multiple forensic objectives simultaneously [12]. Proteomics, the large-scale, quantitative study of proteins, offers several advantages that make it particularly well suited to address these limitations. Proteins are generally more chemically stable than RNA and more informationally diverse than haemoglobin-centred spectroscopic targets [13,14]. The plasma proteome contains thousands of proteins whose abundances are associated with age [15,16], influenced by biological sex and hormonal status [17], and subject to systematic, time-dependent changes after desiccation [18]. Modern high-resolution mass spectrometry platforms operating in data-independent acquisition (DIA) mode can quantify hundreds to more than 1000 proteins from small dried -bloodstain samples, depending on sample quality and workflow depth [19-21]. This review synthesises the current evidence for proteomics-based forensic analysis of blood across three questions: (1) When was this blood deposited? (2) How old was the donor?

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and (3) What is the biological sex of the donor? We describe the analytical workflows required, catalogue the protein markers identified so far, evaluate the machine learning models used to generate predictions, discuss confounders, and propose a roadmap toward integrated, multi-parameter forensic proteomics.

1.1. Scope and Literature Approach

This critical review synthesises peer-reviewed literature on proteomic and complementary molecular approaches to bloodstain analysis, with emphasis on time since deposition, donor age and biological sex estimation. Relevant publications available up to June 2026 were identified through targeted searches of major bibliographic databases, citation chaining and examination of key forensic-proteomics reviews. Priority was given to studies using human blood or bloodstains, quantitative mass spectrometry, independently assessed prediction models, and experimentally characterised environmental or substrate effects. Studies using other matrices or instrumental configurations were included when they informed analytical feasibility, marker biology or workflow design. Because the evidence base remains heterogeneous and limited in cohort size, the review is critical and narrative rather than a formal systematic review or meta-analysis.

2. Analytical Workflow for Dried Bloodstain Proteomics

2.1. Sample Collection and Extraction

The typical starting material for forensic bloodstain proteomics is a disc punched from a dried bloodstain deposited on a substrate such as fabric, tile, wood, paper or glass. Protein yields depend strongly on stain age, substrate porosity and environmental history. Extraction commonly uses 8M urea or 6M guanidinium hydrochloride in Tris-HCl buffer (pH 8.0), followed by sonication to maximise protein solubilisation from the stain matrix [18,21]. A critical consideration is substrate interference: fabrics in particular contribute abundant keratin peptides that can dominate the spectral space and suppress detection of low-abundance proteins. Strategies to mitigate this include protein precipitation (methanol/chloroform or acetone-based), filter-aided sample preparation (FASP), and short-gel electrophoretic pre-fractionation. The input protein amount recoverable from a standard 6 mm punch of a bloodstain typically falls in the range of 1-20 µg depending on stain load, which is compatible with current nano-flow LC-MS/MS systems but constrains the depth of proteome coverage achievable [19,22].

2.2. Proteolytic Digestion

Following protein extraction, samples may be processed by conventional in-solution digestion, filter-aided sample preparation (FASP), or ultrasound-assisted FASP (US-FASP). For in-solution digestion, proteins are reduced with dithiothreitol (10 mM, 56 degrees ^{o}C, 30 min), alkylated with iodoacetamide (20 mM, room temperature, 20 min in the dark), and digested overnight at 37 degrees ^{o}C with sequencing-grade trypsin at an enzyme-to-substrate ratio of approximately 1:50 (*w*/*w*). Alternatively, FASP combines protein digestion with detergent removal and sample clean-up [22]. The protein extract is loaded onto a centrifugal ultrafiltration device, typically with a 30 kDa molecular-weight cut-off, and washed with 8 M urea in Tris buffer. Proteins retained on the membrane are reduced and alkylated, followed by buffer exchange into ammonium bicarbonate or triethylammonium bicarbonate and digestion with sequencing-grade trypsin. US-FASP is a faster alternative in which ultrasonic energy accelerates reduction, alkylation and enzymatic digestion, substantially shortening sample-preparation time while retaining the clean-up advantages of conventional FASP [23]. The resulting peptides are recovered by centrifugation and additional membrane-washing steps. Trypsin predominantly cleaves peptide bonds on the C-terminal side of arginine and lysine residues, except when followed by proline, generating peptides suitable for reversed-phase chromatography and electrospray ionisation. Peptide mixtures obtained with any of these workflows are subsequently desalted using C18 StageTips, micro-solid-phase extraction devices or conventional solid-phase extraction before LC-MS/MS analysis.

2.3. LC-MS/MS Acquisition Strategies

Two broad acquisition strategies are employed in forensic bloodstain proteomics. Data-dependent acquisition (DDA) selects abundant precursor ions for fragmentation in real time. Although it generates high-quality tandem mass spectra and is useful for spectral-library construction, intensity-dependent precursor selection may undersample low-abundance peptides and reduce consistency across analytical runs [20]. On trapped ion mobility spectrometry-time-of-flight (timsTOF) platforms, DDA can be implemented as ddaPASEF, which combines trapped ion mobility separation with parallel accumulation-serial fragmentation to increase sequencing speed and sensitivity, while remaining dependent on precursor selection [24]. Data-independent acquisition (DIA), in which precursor ions within predefined m/z windows are systematically co-isolated and fragmented, provides more comprehensive and reproducible quantification across samples and is therefore particularly attractive for forensic bloodstain studies [19,20]. On timsTOF instruments, DIA can be combined with PASEF in the diaPASEF acquisition mode. This strategy synchronises precursor isolation with ion-mobility separation, adding inverse reduced ion mobility ($1/K_0$) to retention time and m/z, reducing spectral interference and improving sensitivity, quantitative accuracy and reproducibility [25]. These characteristics make diaPASEF a promising option for small or degraded forensic bloodstains, although its application to bloodstain ageing and multi-attribute prediction remains underexplored. The Thermo Scientific Orbitrap Astral, introduced in 2023, has further expanded DIA capabilities through increased acquisition speed, sensitivity and proteome depth. In a recent forensic pilot study, Gao et al. used Orbitrap Astral DIA to analyse bloodstains from 40 male donors stored for four years and identified 1655 proteins, of which 71 were associated with donor age and subsequently evaluated for age-prediction modelling [18].

2.4. Forensic Potential of Ion-Mobility-Enabled Mass Spectrometry

Although LC-diaPASEF has not yet been systematically validated for multi-attribute bloodstain profiling, the forensic applicability of the timsTOF platform has been demonstrated through its MALDI capabilities. Heaton *et al.* used a timsTOF fleX for MALDI profiling, tandem mass spectrometry and imaging of haemoglobin variants in human blood fingermarks, correctly assigning eight of nine samples in a blind set and mapping blood fingermarks at 50 micrometre spatial resolution [26]. Kennedy *et al.* used MALDI MSP and MSI to detect and image human blood in fingermarks on simulated painted walls after Ninhydrin or Acid Black-1 enhancement, confirming haem and haemoglobin peptide markers across depletion series and even detecting blood beneath paint despite polymer interference. Subsequent STR analysis showed DNA profiling remained feasible after MALDI MSI, demonstrating the incorporation capabilities into standard forensic laboratorial analyses [27]. Kennedy *et al.* also applied bottom-up MALDI proteomics to distinguish animal-blood species and provenance, using timsTOF fleX MALDI-MS/MS to confirm markers differentiating blood from wounded animals and packaged meat [28]. Igoh et al. also used a microTOF II for peptide mass fingerprinting and mascot search in order to screen animal species through the analysis of haemoglobin subunits peptides, in both fresh and dried blood samples [29]. In 2025, Kuckova *et al.* applied LC-MS/MS analysis to identify blood present in historical artworks taken from Japanese and Chinese lacquer artefacts dating from the 18th and 19th centuries using an ESI-Q/TOF Maxis Impact, advancing conservation science and addressing protein database limitations [30]. These studies support the broader forensic potential of ion-mobility-enabled instrumentation, while not constituting direct validation of LC-diaPASEF for TSD, age or sex prediction.

2.5. Confounding Variables

A recurring challenge across all three forensic applications reviewed here is the influence of environmental conditions on the protein composition and stability of dried bloodstains. Temperature is the most potent confounder: protein degradation rates increase substantially at elevated temperatures, and TSD models calibrated at 20 °C perform poorly when applied to stains aged at 37 °C [12,31]. Relative humidity affects the oxidation kinetics of haemoglobin, specifically the rate of conversion of methaemoglobin to haemichrome is humidity-dependent, while the initial oxyhaemoglobin-to-methaemoglobin conversion is relatively humidity-independent [9]. UV light exposure and substrate chemical composition introduce additional variability. A combined model trained across all environmental conditions typically achieves markedly lower R^2 than condition-specific models, underscoring the need for environmental metadata in forensic proteomics databases [31]. The main confounding variables are found in **Table 1**.

Table 1 | Principal confounding variables affecting forensic bloodstain proteomics and their implications for predictive-model design.

Variable	Effect on proteome	Relevance for forensic model
Temperature	Accelerates protein denaturation and degradation; shifts TSD kinetics	Must be recorded or estimated from scene; strong confounder for TSD
Humidity	Controls metHb → haemichrome rate; affects peptide solubility on re-extraction	Affects haemoglobin-based TSD but less critical for broad proteome models
Substrate	Introduces exogenous proteins (keratin from fabric); affects extraction yield	Substrate-aware clean-up or interference-control procedures may be required; substrate type should be annotated
UV/light	Photo-oxidation of tryptophan and methionine residues	Indoor vs. outdoor stains require separate calibration models
Donor variability	Individual plasma proteome differs by health status, medication, diet	Cohort diversity essential in training sets; N=40 (Gao *et al.*) still small

3. Time Since Deposition: Blood as a Molecular Clock

3.1. The Haemoglobin Oxidation Cascade

The most studied molecular marker for TSD in blood is haemoglobin, which constitutes approximately 90% of the dry weight of erythrocytes and therefore dominates the dried bloodstain proteome by mass. Upon exit from the vascular system, oxyhaemoglobin (HbO_2, Fe^{2+}, bright red) undergoes biphasic oxidation: a rapid first phase converts HbO_2 to methaemoglobin (metHb, Fe^{3+}, dark red-brown) within hours, followed by a slower second phase converting metHb to haemichrome (HC, dark brown), in which the haem iron coordinates to an additional amino acid ligand [7,9].

The kinetics of the HbO_2 → metHb → HC cascade are strongly temperature-dependent but the metHb → HC transition is additionally sensitive to humidity [9]. These optical changes underlie UV-Vis and Raman spectroscopy-based TSD approaches, which have been reviewed extensively [32,33]. Electrochemical methods, measuring the redox potential of haem species by differential pulse voltammetry, have also been applied over a two-week time series in five temperature conditions, identifying seven of twenty-seven voltammetric variables significantly correlated with TSD ($p < 0.033$) [34]. Although powerful and instrumentation-light, spectroscopic and electrochemical methods are necessarily anchored to a single analyte (haemoglobin) and cannot simultaneously address age or sex.

3.2. Proteome-Wide TSD Markers

The first systematic proteomics-based TSD study was reported by Schneider *et al.* in 2022 using bottom-up DIA LC-MS/MS on dried bloodstains sampled at multiple time points over a two-month period [19]. Principal component analysis (PCA) of the quantified protein matrix showed clear clustering of samples according to TSD. Partial least-squares discriminant analysis (PLS-DA) further confirmed that the proteome composition - not only haemoglobin - encodes temporal information. Key proteins showing systematic abundance changes included components of the complement system, coagulation cascade proteins, and acute-phase reactants, reflecting the progressive degradation and structural reorganisation of the dried blood matrix over time. This work established a critical proof of concept: TSD estimation from blood is not limited to haemoglobin spectroscopy but can be approached with the full proteomic toolkit, opening the door to simultaneous multi-attribute modelling. The two-month observation window, however, leaves longer TSD ranges, relevant to cold cases, unaddressed, and the single-donor, single-temperature study design constrains generalisability [19]. Metabolomics-based TSD work (using LC-HR -MS/MS) has complemented these findings by identifying 28 metabolites with significant circadian oscillations in bloodstains, enabling estimation not only of elapsed time but potentially of time -of-day of deposition [10]. Transcriptomics-based approaches show that mRNA degradation rates support TSD estimation within 2-6 weeks for indoor stains, with accuracy degrading significantly beyond this window [5,6]. Together, these data suggest that a multi -omic approach such as proteomics for longer-range TSD, metabolomics for within-day resolution, and RNA metrics for short -range accuracy, may ultimately be most powerful.

3.3. Protein Degradation Signatures and Chemical Modifications

Changes in protein abundance represent only one component of bloodstain ageing. Following deposition, proteins also undergo a series of spontaneous and environmentally driven chemical modifications that reflect progressive molecular degradation. These include oxidation of susceptible amino acid residues, deamidation, backbone fragmentation and the generation of semi-tryptic peptides, all of which have been recognised as characteristic features of protein ageing in proteomics studies [35,19]. Oxidation is among the earliest detectable modifications in dried blood proteins. Methionine and tryptophan residues are particularly susceptible to reactive oxygen species generated during prolonged exposure to atmospheric oxygen, light and environmental stressors, leading to predictable increases in oxidised peptide species over time [36]. Deamidation of asparagine and glutamine residues proceeds more gradually and has been investigated as a molecular indicator of protein ageing in archaeological, palaeoproteomic and forensic applications, although its dependence on temperature, humidity and protein structure currently limits its use as an independent chronological marker [37]. Progressive protein degradation may also generate endogenous cleavage products that appear as semi-tryptic peptides following bottom-up analysis. Unlike fully tryptic peptides produced during enzymatic digestion, these species reflect cleavage events that occurred before sample preparation and may therefore provide additional information regarding bloodstain ageing [19]. Liu *et al.* have found 4 molecular modifications (succinylation and phosphorylation modification of cystein and the modification of lysine with threonine) with significantly higher values in the old group than the young group, while the carbamylation of lysine was lower in the young group, suggesting an increase in modified proteins at older ages, causing changes in the function of those proteins [38]. DIA workflows are well suited to monitor these molecular changes because modified and unmodified peptide species can be quantified reproducibly across large sample cohorts. Recent advances in library-free database searching and prediction-based spectral matching have further improved the identification of modified peptides without requiring experimentally generated spectral libraries, facilitating more comprehensive characterisation of protein degradation products [39,40]. Consequently, future forensic workflows may benefit from integrating peptide-level degradation signatures with conventional protein abundance measurements to improve the robustness of time-since-deposition models across diverse environmental conditions. Although relatively few forensic studies have systematically evaluated post-translational modifications as direct predictors of TSD, their biochemical basis and compatibility with modern LC–MS/MS workflows make them an important direction for future research. Validation across larger donor cohorts, longer ageing periods and environmentally diverse conditions will be required to determine whether these modifications provide predictive information beyond that obtained from protein abundance alone.

3.4. Machine Learning Models for TSD

TSD estimation has been approached with several statistical frameworks. Linear mixed models have been used in electrochemical studies, treating the biological replicate (individual donor) as a random effect to improve model fit [34]. For proteomics data, dimensionality reduction followed by multivariate regression is more common, with PLS-DA scores along the first latent variable showing linear correlation with elapsed time in the Schneider study [19]. Colorimetric analyses of bloodstains have employed Random Forest regression, achieving R^2 values of up to 0.92 for individual environmental conditions but only 0.71 when all conditions are pooled, a warning that environmental stratification will be essential in any operational model [31].

4. Biological Age Estimation from Bloodstain Proteomics

4.1. The Plasma Proteome as an Aging Clock

The idea that proteins carry an aging signature predates proteomics. It has long been known that certain plasma proteins - such as albumin, immunoglobulins, and complement components - change in concentration with advancing age. The emergence of large-scale mass spectrometry-based plasma proteomics has transformed this insight into a quantitative framework. Studies involving thousands of participants have shown that more than one

-third of detectable plasma proteins change significantly with age [15,41]. Among the most consistently identified aging-associated proteins are GDF15 (a TGF-β superfamily member and hallmark of the senescence-associated secretory phenotype), CXCL9 (an inflammation marker), fetuin-A (hepatokine), and numerous components of the coagulation and complement systems [15,41,42]. Proteomic aging clocks, analogous to the Horvath epigenetic methylation clock, have now been validated in multiple independent cohorts. The ProteAge clock, built on UK Biobank plasma proteomics data, predicts biological age and mortality risk with high accuracy. Critically, proteomic clocks outperform small RNA-based clocks for age prediction when applied to comparable sample sizes [43], an observation directly relevant to the forensic setting where RNA yields from dried stains are often limiting.

4.2. Protein Equalisation

One of the leading challenges in bottom-up proteomics is the presence of highly abundant proteins, which dominate complex biological samples and mask the detection of low-abundance proteins with potential biological or forensic significance [44]. The wide dynamic range of protein concentrations in blood exceeds the analytical capacity of current LC–MS/MS platforms, causing peptides from abundant proteins to preferentially ionize and be selected for fragmentation, thereby reducing proteome coverage and limiting the identification of low-abundance biomarkers [45]. In whole blood, haemoglobin and albumin are two of the most abundant proteins, making them the primary targets for depletion to improve the detection of low-abundance proteins. Several studies have combined abundant protein depletion or equalisation strategies with MALDI-TOF-MS to improve proteomic profiling. Notably, Warden et al, enhanced peptidome profiles with minimal albumin interference and successfully detected the 10.5 kDa protein rKringle5 in plasma after albumin depletion [46]; Fernández *et al.* precipitated disulfide-rich proteins and produced a depleted serum enriched in immunoglobulins [47]; and Araújo *et al.* identified 6 potential protein biomarkers following major protein equalization, consistent with previous reports for bladder cancer [48]. More recently, Domingos *et al.* extended this concept by applying Ultra High-Resolution Quadrupole Time-of-Flight LC-MS/MS analysis to independently investigate both the supernatant and pellet fractions in multiple myeloma samples. This approach highlighted that analysing the high- and low-abundance protein fractions separately provides complementary quantitative proteomic information, with dysregulated proteins from each fraction converging on biological pathways involved in chaperone-mediated autophagy and protein folding [49].

4.3. Proteomic Age Estimation from Dried Bloodstains

The landmark study translating plasma aging proteomics to forensic bloodstains is that of Gao *et al.* [18]. The authors analysed dried bloodstains from 40 healthy male donors spanning an age range of 10-79 years, stored at room temperature for four years, using a Thermo Scientific Orbitrap Astral instrument in DIA mode. A total of 1655 proteins were quantified in bloodstain extracts. Of these, 71 showed significant correlation with donor age (Pearson $|r| > 0.3$, $p < 0.05$): 26 positively correlated and 45 negatively correlated with age. Feature selection was performed using two complementary approaches: LASSO (Least Absolute Shrinkage and Selection Operator) regression, which identified 18 age-associated proteins, and the Boruta algorithm (a Random Forest wrapper), which identified 10. Six proteins appeared in both feature sets, representing the most robustly supported age markers: ITIH3 (inter -alpha-trypsin inhibitor heavy chain 3), HSPA9 (mitochondrial heat shock protein 70), SNAP91 (synaptosomal-associated protein 91), FTL (ferritin light chain), XPO4 (exportin-4), and NCF2 (neutrophil cytosol factor 2). These results are further highlighted in **Table 2**. The best-performing model, a Random Forest trained on the Boruta-selected features, achieved $R^2 = 0.70$ and mean absolute error (MAE) = 9.14 years on an independent test set [18]. These results are promising but must be contextualised. An MAE of approximately nine years is comparable to the current performance of DNA methylation-based forensic age clocks operating on degraded samples, but substantially worse than those achieved with high-quality fresh blood (MAE 2-4 years). The all-male cohort (N = 40) limits inference about sex-related variation in the age proteome, and the four-year storage condition, while realistic for cold-case scenarios, confounds TSD and donor-age effects in a way that future studies must disentangle.

Table 2 | Six proteins identified by both LASSO and Boruta feature selection as age-associated markers in dried bloodstains in Gao *et al.* [18].

Protein	Full name	Direction with age	Biological relevance
ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3	Negative	Acute-phase reactant; hyaluronan matrix stabilisation; decreases with chronic inflammation of aging
HSPA9	Mitochondrial Hsp70 / mortalin	Positive	Mitochondrial chaperone; accumulates with oxidative stress in aging cells
SNAP91	Synaptosomal-associated protein 91	Negative	Involved in clathrin-mediated endocytosis; altered neuronal function with aging
FTL	Ferritin light chain	Positive	Iron storage; elevated with aging; oxidative stress marker
XPO4	Exportin-4	Negative	Nuclear export of eIF5A and Smad3; links to senescence pathways
NCF2	Neutrophil cytosol factor 2	Positive	NADPH oxidase component; increased reactive oxygen species production in aging neutrophils

4.3. Comparisson with other Biological Age Estimation Approaches

DNA methylation clocks (Horvath, GrimAge, PCPhenoAge) represent the current reference standard for forensic age estimation, with MAE values of 3-5 years achieved on fresh blood. Their performance on aged or damaged DNA is, however, substantially worse, and the requirement for bisulphite conversion introduces additional degradation of already compromised material [50]. Using a multiplex methylation SNaPshot assay, Dias et al. developed an age prediction model based on four CpG sites (ELOVL2, FHL2, C1orf132 and TRIM59) that explained 92.5% of the variation in chronological age (adjusted R^2 = 0.925) with a MAD of 4.97 years across combined blood samples from living and deceased individuals [51]. Telomere length analysis, while feasible from bloodstains, provides population-level age trends rather than individual-level predictions and has high inter-individual variability [50]. RNA-based age clocks are impractical for long TSD stains due to RNA instability. Proteomics-based age estimation occupies a complementary niche: proteins are more stable than both RNA and methylated DNA under adverse storage conditions, the approach is compatible with the same LC-MS/MS workflow used for TSD and sex analysis (enabling multi-attribute analysis from a single extraction), and the protein panel can be continuously refined as larger forensic cohorts are studied. The main current limitations are the relatively small training cohorts published to date and the need to account for TSD-related protein changes that may confound age signals.

5. Biological Sex Estimation from Blood Proteomics

5.1. Protein Markers for Biological Sex in Blood

Biological sex leaves a strong imprint on the plasma proteome through several mechanisms: (i) differential expression of Y-chromosome-encoded proteins absent in females; (ii) hormonal regulation of liver-produced secreted proteins; and (iii) immune and inflammatory protein expression differences that are consistent across populations. Testosterone, oestrogen, and their receptors modulate the transcription of hundreds of genes encoding plasma proteins, many of which are quantifiable by LC-MS/MS [17,52]. The most studied sex-specific protein in forensic contexts is amelogenin, which produces two isoforms encoded by the X (AMELX) and Y (AMELY) chromosomes. AMELY detection in enamel peptides has enabled proteomic sex determination from teeth and bone in archaeological remains, and the approach has been validated on heavily degraded material [53]. However, amelogenin is not expressed in blood, making it inapplicable to bloodstain analysis. For blood specifically, the 2025 study by Alex *et al.* provides the most detailed characterisation, translated to **Table 3** [17]. The authors analysed LC-MS/MS proteomes from 100 whole blood samples of known biological sex (50 male, 50 female) and applied gradient-boosting classifiers (with L1 and L2 regularisation, analogous to LASSO and ridge regression). Cross-validated classification achieved high overall accuracy, with pregnancy zone protein (PZP) and ceruloplasmin (CP) peptides among the most discriminating features. PZP is normally expressed in high amounts only during pregnancy but shows residual sex-differential expression in non-pregnant females at steady state [54]; CP is higher in females due to oestrogen-mediated transcriptional upregulation [55].

5.2. Challenges and Limitations

Despite these promising markers, the Alex *et al.* study explicitly cautions that proteomics-based sex estimation from blood faces technical and biological obstacles that may limit its forensic utility [17]. Principal among these is the very large inter-individual variability in plasma protein concentrations - driven by health status, medication use, hormonal contraception, age, and diet - that partially obscures sex-related differences. The dynamic range of the blood proteome spans more than ten orders of magnitude, with highly abundant proteins (haemoglobin, albumin, immunoglobulins) routinely suppressing detection of low-abundance sex-specific markers [56]. An additional concern is the effect of TSD on sex-discriminating proteins. If stain age differentially affects the stability of female- versus male-enriched proteins, a classifier trained on fresh blood will give biased predictions when applied to aged stains. To date, no study has systematically addressed this interaction. The authors of the sex estimation study therefore recommend that forensic proteomics research prioritise source attribution (identifying the body fluid and tissue of origin) and TSD estimation - where proteomic signals

Table 3 | Key proteins proposed for biological-sex estimation in forensic proteomics, with mechanisms and forensic applicability.

Protein	Sex enrichment	Mechanism	Forensic utility
Pregnancy zone protein (PZP)	Female	Oestrogen-regulated; elevated in pregnancy but detectable at lower levels in non-pregnant females	High sensitivity, moderate specificity; confounded by hormonal status
Ceruloplasmin (CP)	Female	Oestrogen upregulates hepatic CP expression via transcription factor binding	Widely quantified in DIA workflows; moderate sex-ratio
Sex hormone-binding globulin (SHBG)	Female > Male	Regulates free testosterone; higher in females and in males with low testosterone	Useful but affected by age and BMI
Prostate-specific antigen (PSA, KLK3)	Male	Tissue-specific to prostate; detectable in blood at low ng/mL range	Useful in aged males; absent in females
Amelogenin-Y (AMELY)	Male (tissue)	Y-chromosome-encoded enamel protein	Validated in bone/tooth; not expressed in blood

are stronger and more reproducible - over phenotypic profiling of donors, where the gain above existing immunological and DNA-based methods is currently modest [17].

6. Integration: Toward a Multi-Attribute Forensic Proteomic Workflow

6.1. Simultaneous Multi-Parameter Prediction

The three forensic attributes reviewed here, SD, donor age and biological se, are not analytically independent. Signals potentially informative of all three attributes may be recoverable from the same proteomic dataset generated from a single LC-MS/MS analysis of a bloodstain extract, although simultaneous prediction has not yet been prospectively demonstrated in one validated bloodstain cohort. This creates both an opportunity and a statistical challenge. A unified workflow could preserve material by producing several investigative outputs from one punched disc. However, potentially informative features may overlap: age-associated proteins such as FTL and HSPA9 may also change with TSD; sex-associated proteins such as PZP may vary with age and hormonal status; and TSD-associated acute-phase proteins may be influenced by both age and sex [13,17,18]. Multi-output machine learning frameworks, such as multi-task Random Forest, multi-response LASSO, or deep learning architectures trained on shared latent representations, offer a principled way to jointly model all three attributes while accounting for their dependencies. Such frameworks have been applied in clinical proteomics but have not yet been deployed in forensic bloodstain research, representing a clear priority for the field.

6.2. Machine Learning Approaches

Several machine learning approaches have been applied across the studies reviewed for forensic blood proteomics. Random Forest regression and classification have been used for age estimation and TSD modelling [18,31], offering robustness to outliers and non-linear relationships while handling high-dimensional proteomics data through feature importance scoring. LASSO and elastic-net regression have been employed for age marker selection and sex classification [17,18], performing simultaneous feature selection and regularisation with interpretable coefficients that map directly to protein contributions. PLS-DA has been applied to visualise proteome-wide separation among predefined TSD groups [19], whereas continuous elapsed-time prediction requires regression approaches such as PLS regression. Gradient boosting methods, including XGBoost and LightGBM with L1/L2 regularisation, have been applied to sex classification [17] and generally outperform Random Forest on tabular data when sample sizes are sufficient. Finally, linear mixed models have been used in electrochemical and RNA-based TSD studies [11,34], and are appropriate when repeated measures from the same donor are available, with donor treated as a random effect. Nevertheless, quantitative proteomics data require extensive preprocessing before statistical modelling to minimise technical variation while preserving biologically meaningful differences. Protein intensities are typically normalised using median, total-ion-current or variance-stabilising approaches to reduce systematic differences arising from sample preparation and LC-MS/MS performance [57]. Missing values, which commonly result from stochastic peptide detection or low-abundance proteins, must be handled carefully because inappropriate imputation can introduce bias into downstream prediction models [58,59]. Frequently applied strategies include filtering proteins with excessive missingness, imputing values based on local similarity or low-abundance distributions or employing machine-learning algorithms capable of accommodating incomplete datasets directly [59]. Prior to model training, protein identifications are generally filtered using a peptide- and protein-level false discovery rate (FDR) of 1%, with quantification based on proteotypic peptides consistently detected across samples [60]. Where analyses are performed over extended acquisition periods or across multiple batches, batch-effect correction and quality-control monitoring using pooled reference samples are recommended to ensure analytical reproducibility and minimise instrument-related variation [59,61]. These preprocessing steps are well established in quantitative proteomics and are essential to produce robust forensic prediction models with reproducible performance across laboratories and analytical platforms. For DIA workflows, software platforms such as DIA-NN, and FragPipe implement many of these preprocessing procedures, including interference correction, protein inference, normalisation and false-discovery-rate control, thereby generating quantitative protein matrices suitable for downstream statistical and machine-learning analyses [40,62]. A consistent limitation across all published models is the small size of training cohorts, typically fewer than 100 individuals. Given the high within-population variability of plasma protein concentrations, reliable model training for forensic applications will likely require datasets of several hundred to several thousand individuals. The establishment of open, curated forensic proteomics reference databases - analogous to existing DNA STR frequency databases - is therefore an urgent priority for the field.

6.3. Proposed Workflow

Based on the evidence reviewed, and as depicted in **Figure 1**, we propose the following integrated workflow for multi-attribute forensic bloodstain proteomics using trapped ion mobility spectrometry coupled to high-resolution mass spectrometry:

1. **Scene documentation:** Photograph, measure, and record the stain substrate, estimated stain dimensions, sampling location, and relevant environmental conditions, including temperature, humidity, light exposure, and potential UV exposure history, when determinable.
2. **Stain extraction:** Excise one or more 6 mm discs from the bloodstain. Extract proteins in 8M urea buffer using brief probe or bath sonication. Determine protein concentration using a BCA assay and normalise the input to a constant protein mass whenever sufficient material is available.
3. **Tryptic digestion:** Reduce disulfide bonds, alkylate cysteine residues, and digest proteins with sequencing-grade trypsin.

Desalt the resulting peptides using C18 micro-solid-phase extraction, dry under vacuum, and reconstitute in 0.1% formic acid.

4. **nanoLC-timsTOF analysis:** Separate peptides by nano-flow reversed-phase liquid chromatography at approximately 300 nL/min using a 60-90 min gradient. Analyse peptides using a timsTOF instrument operated in diaPASEF mode, combining high-resolution mass spectrometry with trapped ion mobility separation. Optimise the ion-mobility range, collision-energy ramp, precursor isolation scheme, accumulation time, and duty cycle for blood-derived peptide mixtures.
5. **Ion-mobility-aware data processing and protein quantification:** Process diaPASEF data using software compatible with Bruker trapped ion mobility data, such as DIA -NN, Spectronaut, or FragPipe. Perform either library-free analysis using predicted spectra and ion-mobility values or library-based analysis using a project-specific spectral library generated from pooled reference blood samples analysed by ddaPASEF or diaPASEF. Protein quantification should be based on consistently detected proteotypic peptides, with a minimum of two unique or protein-group-specific peptides per reported protein. Apply appropriate between-run normalisation and control peptide- and protein-level false-discovery rates at 1%.
6. **Four-dimensional feature extraction:** Integrate peptide intensity, retention time, mass-to-charge ratio and inverse reduced ion mobility ($1/K_0$). Where appropriately derived or calibrated, collisional cross-section values may also be used. Ion mobility should serve as an additional identification and quality-control dimension to improve discrimination between co-eluting peptides, reduce interference and assess analytical consistency across stains, substrates and ageing conditions.
7. **Multi-attribute modelling:** Apply pre-validated Random Forest, LASSO, or other suitable multivariate models for time since deposition, donor age, and biological sex in parallel. Models may incorporate protein abundance, selected peptide abundance, and ion-mobility-derived variables. Generate point predictions, class probabilities, and confidence or prediction intervals, and cross-reference outputs to assess internal consistency among the predicted attributes.
8. **Quality control:** Include pooled blood reference samples, extraction blanks, digestion blanks, retention-time or ion-mobility reference standards, and replicate injections. Monitor peptide identifications, protein-group numbers, precursor intensity, mass accuracy, retention-time stability, ion-mobility stability, missing-value rates, and carryover. Samples failing predefined analytical criteria should be flagged or excluded from predictive modelling.
9. **Reporting:** Report point estimates, confidence or prediction intervals, model probabilities, and analytical reliability flags. Relevant limitations should include low protein yield, high levels of missing data, poor digestion efficiency, substrate-related interference, environmental conditions outside the model training range, and ion-mobility or retention-time deviations from quality-control limits.

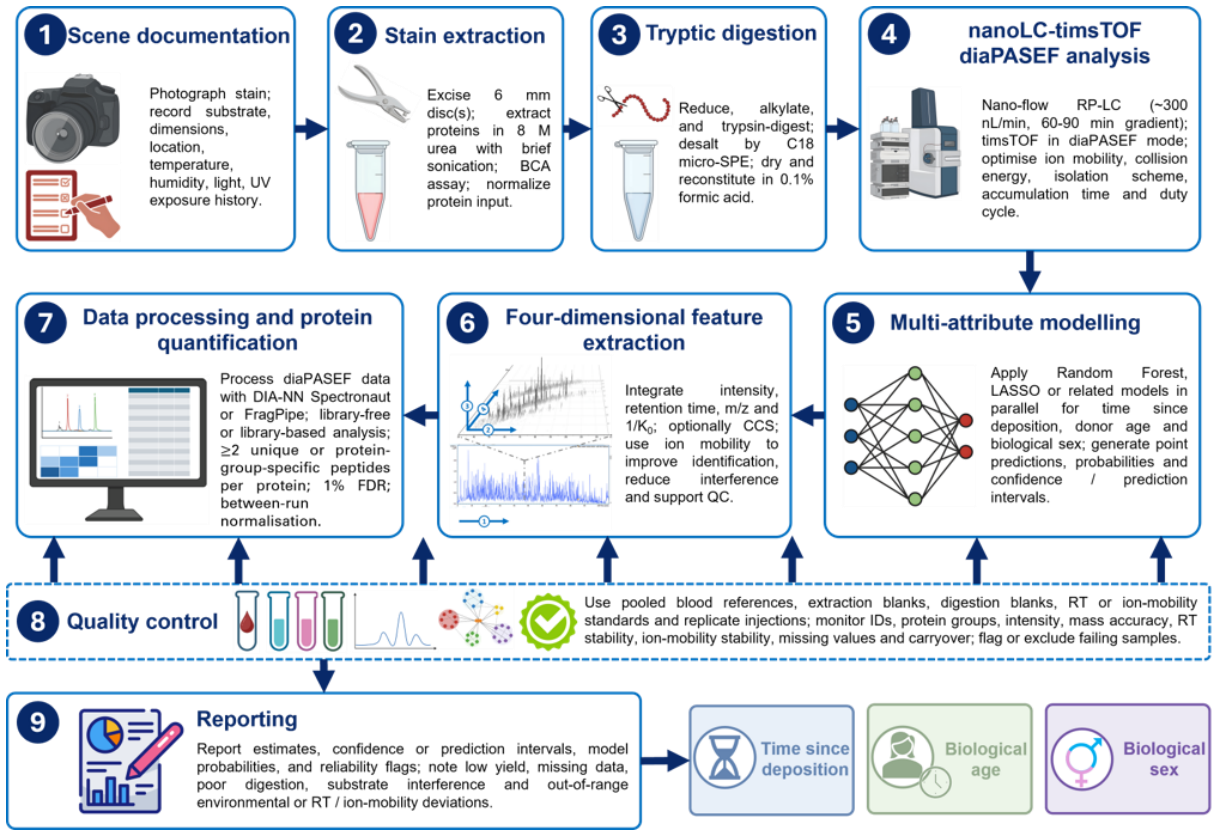

Figure 1 | Proposed workflow for multi-attribute forensic bloodstain proteomics.

7. Current Challenges and Future Directions

Several challenges must be addressed before forensic bloodstain proteomics reaches operational maturity:

7.1. Cohort size and diversity

All published forensic bloodstain proteomics studies have cohort sizes below 100 individuals, and several are limited to a single sex or narrow age range. Larger, population-diverse cohorts are needed, and reference datasets should span a range of health conditions, medications, and ethnicities to avoid systemic bias in predictions.

7.2. Disentangling TSD from donor age

Both TSD and donor biological age affect the bloodstain proteome, but in potentially overlapping ways. A study design that varies both dimensions independently, collecting stains from donors across the age spectrum and ageing each stain across multiple time points, is needed to build models that can deconvolve these two effects.

7.3. Environmental calibration

Protein degradation kinetics are non-linearly dependent on temperature and humidity. Stains recovered from outdoor scenes, vehicles, or tropical climates will have experienced very different environmental histories than laboratory-aged standards. Environmental sensor data co-deposited at scenes (temperature loggers, humidity records) or computational reconstruction from meteorological data could partially address this, but calibration across a wider range of conditions is required.

7.4. Substrate diversity

Most published studies use cotton fabric, glass, or filter paper as substrates. Real crime scenes involve wool, synthetic textiles, carpet, soil-contaminated surfaces, and mixed-substrate stains. Each substrate introduces different extraction efficiencies, different levels of contaminant proteins, and different physical protection of stain proteins from environmental degradation.

7.5. Legal and ethical framework

Inferring age, sex, or phenotypic characteristics from biological traces raises significant legal and ethical questions, particularly regarding the permissible scope of forensic intelligence. Thus, distinguishing information used to generate investigative leads from evidence admissible in court. Regulatory frameworks for forensic DNA phenotyping are still evolving in most jurisdictions, and proteomics-based phenotyping will require equivalent debate and legislation.

7.6. Integration with existing forensic workflows

Forensic laboratories operate under strict accreditation requirements (ISO/IEC 17025:2017), and any new analytical method must be validated according to SWGMAT or equivalent standards before operational use. This includes establishing limits of detection, specificity, precision, accuracy, and robustness across the intended operational range of conditions.

8. Conclusions

Mass spectrometry-based proteomics has emerged as a credible and powerful platform for the multi-attribute forensic analysis of bloodstains. The three principal attributes reviewed here - time since deposition, biological age, and biological sex - are each supported by proof-of-concept evidence and can, in principle, be investigated within a single integrated analytical workflow. In the approach proposed here, the analytical core is based on nanoLC coupled to timsTOF mass spectrometry operating in diaPASEF mode, which combines data-independent acquisition with trapped ion mobility separation. This four-dimensional framework, incorporating mass-to-charge ratio, retention time, ion mobility, and signal intensity, offers increased precursor selectivity, reduced spectral interference, and improved analytical reproducibility for complex and potentially degraded forensic samples. The required informatics infrastructure is also increasingly mature. Software platforms such as DIA-NN, Spectronaut and FragPipe can process diaPASEF data using project-specific spectral libraries or library-free prediction strategies, while established statistical and machine-learning environments support multivariate modelling of forensic attributes. Although the analytical feasibility of deep and reproducible bloodstain proteomics is increasingly well established, a unified diaPASEF-based multi-attribute assay still requires direct experimental validation. Larger and more diverse donor cohorts, systematic assessment of environmental and substrate effects, harmonised quality-control criteria and genuinely multi-attribute study designs are now required. The six proteins consistently associated with donor age in dried bloodstains - ITIH3, HSPA9, SNAP91, FTL, XPO4, and NCF2 - provide a rational starting panel for the development of proteomic age-estimation models. The feasibility of estimating time since deposition using proteome-wide DIA measurements has also been demonstrated, although future studies must extend the validated time range and account for temperature, humidity, UV exposure, substrate type, and other environmental variables. Biological sex estimation using blood proteomics remains less accurate than established genetic approaches, but it may still provide useful complementary information as one component of a broader multi-parameter forensic profile. The addition of trapped ion mobility is particularly relevant for forensic applications because it provides an orthogonal dimension for peptide identification and quality control. Ion-mobility or collisional-cross-section information may improve the discrimination of co-eluting species, increase confidence in peptide assignments, and support the detection of analytical drift or matrix-related interference. However, ion-mobility-derived variables must themselves be rigorously validated across instruments, laboratories, substrates, and ageing conditions before they can be incorporated into forensic prediction models. Forensic proteomics is not currently positioned to replace DNA profiling for individualisation. Its value lies instead in complementing genetic analysis by

providing contextual information about when a bloodstain was deposited and about broad biological characteristics of the donor. A validated timsTOF-based diaPASEF workflow could therefore contribute to crime-scene reconstruction beyond the question of identity. With appropriate investment in reference-cohort development, environmental modelling, inter-laboratory validation, standardisation, and machine-learning integration, multi-attribute bloodstain proteomics may become a practical component of forensic investigation over the coming decade.

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