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EDITED BY

Jan Halamek,
Texas Tech University, United States

REVIEWED BY

Flaminia Vincenti,
Sapienza University of Rome, Italy
Alicja Menżyk,
University of Silesia of Katowice, Poland

*CORRESPONDENCE

Theresa E. Stotesbury,
theresa.stotesbury@ontariotechu.ca

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The crux of time: A meta-analysis of ex vivo whole blood degradation

Colin I. Elliott¹, Aaron B. A. Shafer^{2,3} and Theresa E. Stotesbury^{4*}

¹Forensic Chemistry and Materials Lab, Applied Bioscience Graduate Program, Ontario Tech University, Oshawa, ON, Canada, ²Wildlife and Applied Genomics Lab, Department of Forensic Science, Trent University, Peterborough, ON, Canada, ³Wildlife and Applied Genomics Lab, Environmental and Life Sciences Graduate Program, Trent University, Peterborough, ON, Canada, ⁴Forensic Chemistry and Materials Lab, Faculty of Science, Forensic Science, Ontario Tech University, Oshawa, ON, Canada

Estimating the time since deposition (TSD) of a bloodstain can provide important medico-legal information for crime scene investigation. Research in this area primarily investigates the degradation of either hemoglobin or genetic material over time. In this work, we present a comprehensive meta-analysis on bloodstain TSD research. Our results are interpreted from 25 quantitative studies used to probe the effect of biomolecule studied, analytical technique used, substrate porosity, environmental conditions, and blood source on TSD estimates. There was an overall strong effect of time across studies (Fisher's $Z_r = 1.66$, $r = 0.93$), and generally, we found that the type of biomolecule studied (e.g., hemoglobin, DNA) had equal effect sizes for TSD estimation. Differences in the mean TSD effect size were also observed between substrate porosity. Interestingly, the blood source does not significantly influence the magnitude of the effect sizes in TSD estimation. Despite the clear effect of time, forensically relevant prediction of bloodstain TSD remains complicated by inter-donor variability, type of substrate and environmental conditions. We recommend that future bloodstain TSD research increase sample size, include summary statistics and standardize experimental methodologies so that we can develop a quantitative understanding of the physicochemical processes involved in whole blood degradation in ex vivo conditions.

KEYWORDS

forensic chemistry, applied genomics, time since deposition (TSD), bloodstains, spectroscopy, biomolecules, substrate porosity

Introduction

Crime scene investigators are tasked with determining the events of a crime. This normally consists of answering forensically relevant questions like *who*, *what*, *where*, *how*, and, *when*, regarding the observed and collected evidence at the crime scene. Biological material such as tissue and bodily fluids left at a crime scene can assist in answering these questions. A commonly encountered type of biological evidence at a crime scene is blood (Box 1). As blood dries, it forms bloodstains with a variety of morphologies largely

depending on formation mechanics and target surface properties (Adam, 2012). Source identification of a bloodstain is conducted through DNA profiling (Butler, 2006; Verdon et al., 2011, 2013) while bloodstain pattern analysis (BPA) provides potential interpretations of the physical events that created the bloodshed (Adam, 2012).

Both DNA profiling and BPA are important to an investigation, yet they do not provide an estimate of *when* the crime occurred. Determining the amount of time that has elapsed since a bloodstain was deposited, or time since deposition (TSD), could provide important context to investigators. This is especially useful in cases where blood is the only biological evidence present; a timeline could be established and used to corroborate physical evidence, witness testimonies and the suspect alibis (Bremmer et al., 2012; Zadora and Menzyk, 2018). Alternatively, TSD can be used to determine whether a bloodstain is relevant to the current investigation (Zadora and Menzyk, 2018). Bloodstain TSD is typically estimated by modelling the physicochemical degradation of blood biomolecules over time; experimental methodologies generating spectroscopic (Hanson and Ballantyne, 2010; Doty et al., 2016; Zhang et al., 2017), genetic (Anderson et al., 2011; Fang et al., 2019; Salzmann et al., 2021b) and photographic (Thanakiatkrai et al., 2013; Shin et al., 2017) metrics have been developed and used to estimate bloodstain TSD. Factors like individual variation,

type of substrate and environmental conditions influence the timewise degradation of bloodstains and their biomolecules, resulting in large variability in the accuracy and precision of TSD estimation models (Zadora and Menzyk, 2018; Weber and Lednev, 2020; Menzyk, 2021).

Commonly analyzed biomolecules for TSD estimation models include hemoglobin (Hb) from red blood cells (RBCs) and genetic material from white blood cells (WBCs). Spectroscopic techniques, such as Raman (Premasiri et al., 2012; Doty et al., 2016, 2017; Sun et al., 2017b; Takamura et al., 2019; Menzyk et al., 2020), infrared (Botoniic-Sehic et al., 2009; Lin et al., 2017; Zhang et al., 2017; Hassan et al., 2019), reflectance (Bremmer et al., 2011a, 2011b; Li et al., 2011; Edelman et al., 2012a, 2016; Sun et al., 2017a) and UV-Vis absorbance (Hanson and Ballantyne, 2010; Hanson et al., 2011; Agudelo et al., 2015; Bergmann et al., 2017, 2021; Kaur et al., 2020; Stotesbury et al., 2020; Cossette et al., 2021) spectroscopies have all probed the timewise degradation of Hb. Photography (Thanakiatkrai et al., 2013; Shin et al., 2017; Choi et al., 2019) and hyperspectral imaging (Edelman et al., 2012b; Li et al., 2013; Majda et al., 2018) have measured the colour changes in blood while electron spin resonance and electrochemistry analyzed the conformational changes of the heme groups (Miki et al., 1987; Matsuoka et al., 1995; Fujita et al., 2005). Atomic force microscopy has investigated the changes in RBC physiology over time (Strasser et al., 2007;

BOX 1

Blood is a non-Newtonian fluid responsible for the maintenance of homeostasis *in vivo* by providing transport of oxygen, carbon dioxide, minerals, and electrolytes, among others, to and from every part of the human body. Plasma, which makes up approximately 55% of whole blood, is mostly water but does contain some proteins, minerals, and electrolytes while the cellular components, which make up the remaining 45% of whole blood, include erythrocytes (red blood cells; RBC), leukocytes (white blood cells; WBC) and thrombocytes (platelets) (Bremmer et al., 2012). The cellular components and plasma proteins perform specific physiological functions *in vivo* that are disrupted when the blood leaves the body (*ex vivo*).

Hemoglobin (Hb), an important protein in RBCs that is responsible for the transport of oxygen in the body, consists of two α and two β chains (Zadora and Menzyk, 2018). Each subunit or chain contains a heme group, with a centralized iron atom in a protoporphyrin ring, directly capable of reversibly binding oxygen (Bergmann et al., 2021). Oxygen-bound hemoglobin is referred to as oxyhemoglobin (oxyHb) while Hb without oxygen is referred to as deoxyhemoglobin (deoxyHb) (Bergmann et al., 2021). *In vivo*, every day roughly 3% of oxyHb is autoxidized to methemoglobin (metHb), a derivative incapable of binding and carrying oxygen (Bonaventura et al., 2013; Zadora and Menzyk, 2018). In deoxyHb, iron is present in its ferrous state (Fe^{2+}); upon oxidation to oxyHb, the iron is in either its ferrous or ferric state (Fe^{3+}) (Yamamoto and Palmer, 1973; Spiro and Strekas, 1974; Kitagawa, 1987; Ferraro et al., 2003; Nagatomo et al., 2011). This differs from the autoxidized metHb in which iron is solely present in its ferric state (Doty et al., 2016; Zadora and Menzyk, 2018; Bergmann et al., 2021). *In vivo*, internal reduction mechanisms including enzymes glutathione peroxidase and methemoglobin reductase are responsible for maintaining low levels of metHb by converting Hb to its healthy derivative (Bonaventura et al., 2013; Zadora and Menzyk, 2018). *Ex vivo*, when blood is deposited, the combination of environmental stressors and loss of the internal reduction mechanisms lead to accelerated oxidative changes in Hb derivatives (Bonaventura et al., 2013; Doty et al., 2016; Zadora and Menzyk, 2018). Continued degradation creates further conformational changes to the structure of Hb, leading to the formation of hemichromes (HCs) (Bergmann et al., 2021). The iron in this Hb derivative binds a distal histidine residue rather than oxygen or water (Doty et al., 2016; Zadora and Menzyk, 2018). This *ex vivo* degradation pathway has been of great interest to bloodstain TSD research due to the continuous structural and redox changes in the Hb (Weber and Lednev, 2020).

Other important molecules in blood include DNA and RNA present in WBCs; DNA is double-stranded while RNA is single-stranded and can form extensive secondary and tertiary structures (Nowakowski and Tinoco, 1997; Fordyce et al., 2013). RNA encompasses larger ribosomal RNA and the shorter messenger RNA (mRNA) and micro RNA (miRNA), among others (Wu et al., 2014). *Ex vivo*, DNA and RNA are both degraded similarly; enzymes cleave their phosphodiester bonds most effectively in hydrated environments (Fordyce et al., 2013), which occurs before desiccation of the bloodstain. Reactive oxygen species, alkylating agents and UV irradiation damage the nucleotides and can induce crosslinking, pyrimidine dimers and chain breaks (Wurtmann and Wolin, 2009; Fordyce et al., 2013; Rahi et al., 2021). As a result, base-pairing is disrupted, leading to fragmentation and unstable molecules. However, shorter RNAs like mRNA and miRNA have been shown to persist for long periods in desiccated environments (Fordyce et al., 2013; Salzmann et al., 2021b), suggesting that the degradation means suggested above are biased towards larger fragments of nucleic acid. Although RNA might provide insight for long-term studies and complement the short-term degradation of hemoglobin, they have yet to be combined to estimate bloodstain TSD.

Wu et al., 2009; Cavalcanti and Silva, 2019) while mass-spectrometry (Seok et al., 2018; Rankin-Turner et al., 2019), chromatography (Inoue et al., 1991, 1992; Andrasko, 1997; Arany and Ohtani, 2011; Acar et al., 2020) and fluorescence spectroscopy (Guo et al., 2013; Mc Shine et al., 2017; Weber et al., 2021) have measured changes in metabolites and protein composition of deposited bloodstains. DNA (Kohlmeier and Schneider, 2012; Sirker et al., 2013, 2016; Rahikainen et al., 2016; Stotesbury et al., 2020; Cossette et al., 2021), total RNA, mRNA and miRNA (Bauer et al., 2003; Anderson et al., 2005, 2011; Simard et al., 2012; Qi et al., 2013; Alshehhi et al., 2017; Fang et al., 2019; Fu and Allen, 2019; Mayes et al., 2019; Alshehhi and Haddrill, 2020; Asaghiar and Williams, 2020; Salzmann et al., 2021b; Heneghan et al., 2021; Li et al., 2021; Manasatienjig and Nimmual, 2021; Zhao et al., 2021) degradation have also been targeted in longer time series. Recently, microbial profiling of bloodstains has described the timewise changes in microbial communities inhabiting the bloodstains (Salzmann et al., 2019; 2021a). Most of these works are summarized in greater depth in the recent reviews by Zadora and Menzyk and Weber and Lednev (Zadora and Menzyk, 2018; Weber and Lednev, 2020). Interestingly, some studies have attempted to determine the time of day of deposition by evaluating circadian biomarkers (Ackermann et al., 2010; Lech et al., 2016, 2018) and the age of the donor by examining changes in methylation (Zbieć-Piekarska et al., 2015; Thong et al., 2017) or signal joint T-cell receptor rearrangement excision circles (Ou et al., 2012; Yamanoi et al., 2018).

The type of substrate on which the bloodstain is deposited also influences TSD estimates due to differing degradation pathway kinetics (Zadora and Menzyk, 2018). The rate of biomolecule degradation in bloodstains is partially dictated by substrate porosity; for example, hemoglobin oxidation is a faster process in dried states than in liquid states (Marrone and Ballantyne, 2009). Substrates range from common materials like glass, plastic and textiles to less common materials like soil (Hassan et al., 2019), wallpaper (Shin et al., 2017), leather (Kohlmeier and Schneider, 2012) and brick stones (Wang et al., 2019). The same applies to environmental conditions; warmer environments catalyze biomolecular degradation while colder environments preserve the biomolecules in their native states, slowing the degradation (Zadora and Menzyk, 2018). A variety of environmental conditions have been studied in bloodstain TSD research; this includes exposure to outdoor environments (Lin et al., 2017; Salzmann et al., 2021a, 2021b; Li et al., 2021; Zhao et al., 2021), varying temperatures (−20–61°C) and a wide range of humidity values (10–99% RH) (Hanson and Ballantyne, 2010; Mayes et al., 2019; Cossette et al., 2021; Heneghan et al., 2021; Li et al., 2021; Zhao et al., 2021). Statistical analyses also differ between studies; regressions are common, but the type of fit varies (linear (Fu and Allen, 2019), polynomial (Cavalcanti and Silva, 2019), logarithmic (Sun et al., 2017b), exponential (Gautam et al., 2020), multivariate (Cossette et al., 2021)) depending on the

observed time-series trends. A number of studies used dimensionality-reduction tools for their analyses (Sun et al., 2017a; Doty et al., 2017) while others incorporated cross-validation to obtain prediction accuracies (Bremmer et al., 2011b; Edelman et al., 2012b). The inter-study variability in experimental conditions and statistical analyses raises two fundamental questions: how do we interpret all this research together and what is the best way forward for bloodstain TSD research?

We present a meta-analysis that examines the association between the degradation of biomolecules and cellular elements found within blood and its TSD. The primary goals here are to determine the strength of these relationships and whether certain analytical techniques and biomolecules provide more robust blood TSD estimates. Specifically, we 1) determined the mean effect size—a quantitative value measuring the strength of a relationship—of bloodstain TSD studies, 2) explored how effect sizes varied by class of biomolecule, analytical technique, substrate porosity, blood source, temperature, and type of summary statistic, and, 3) searched and analyzed compiled data for a publication bias. By using a meta-analytic approach, we synthesized quantitative data on blood TSD estimates, complementing recent qualitative reviews (Bremmer et al., 2012; Zadora and Menzyk, 2018; Weber and Lednev, 2020). The combination of quantitative and qualitative data provides a holistic overview and way forward for bloodstain TSD research and ultimately, crime scene implementation.

Materials and methods

This meta-analysis was conducted using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for reporting articles in systematic reviews and meta-analyses (Page et al., 2021b). The process for this reporting system is normally reflected in a step-by-step flowchart indicating the number of articles that were screened for inclusion in the meta-analysis.

Literature search and strategy

Literature searches were conducted in two databases: Web of Science and Scopus. The searches included full-text articles, summaries, and abstracts. Searches were conducted in English and did not include a restriction for the year of publication. Following the searches, which were completed 12 May 2021, databases were searched using a combination of the following keywords: “DNA,” “RNA,” “time since deposition,” “bloodstains,” “hemoglobin,” “taphonomy,” “blood,” “degradation,” “forensic,” “spectroscopy,” “metabolites,” “proteins,” “plasma proteins,” “aging,” “volatile organic

compounds,” “transcriptome,” “methylation.” The combination of keywords was done through the Boolean operator “AND” and resulted in 26 searches. The number of hits for each search is found in [Supplementary Table S1](#). Hits were imported into EndNote citation manager for eligibility assessment.

Eligibility criteria and data collection

Articles were first screened by title, followed by abstracts and finally full text. Articles selected were peer-reviewed English publications quantitatively examining the TSD of blood. The following articles were excluded: 1) commentaries, editorials, reviews, opinions; 2) articles examining the TSD of other biological fluids, 3) articles providing a qualitative assessment of blood TSD in which there were no convertible summary statistics (r , R^2 , F), 4) articles in which blood was not deposited on a substrate, 5) articles not in English; and 6) articles where the full text was not available. Data from retained articles were extracted and inserted into a summary table. Data collected, if reported, included summary statistics (r , R^2 , F , t , p -value), the number of biological replicates, the class of biomolecule examined (whole blood and cellular elements, genetic material, proteins), the analytical technique used to analyze the timewise trends, blood source (e.g., human, bovine), the temperature, substrate porosity, length of time series, and author affiliation (country).

Three-level meta-analysis

All statistical analyses were conducted in R version 4.0.3. The R script and datasets for this study are publicly available and deposited at “<https://gitlab.com/f4301/TSD.git>”. Reported summary statistics from each article were first converted to the correlation coefficient r if not provided in the texts and used to calculate effect size (Fisher’s Z_r) and standard error (SE_z) with the following formulas (Fisher, 1921):

$$Z_r = 0.5 \log_e \frac{(1+r)}{(1-r)} \quad (1)$$

and

$$SE_z = \frac{1}{\sqrt{n-3}}, \quad (2)$$

where n is the sample size, represented by the number of biological replicates. Summary statistics reported in the retained articles included r , R^2 , and p -values. For studies reporting R^2 , conversion to r was completed using the following:

$$r = \sqrt{R^2 - \frac{i(1-R^2)}{n-i-1}}, \quad (3)$$

where i is the number of independent variables in the regression model (e.g., time and/or temperature) (Nakagawa et al., 2007). Effect sizes were only computed from models evaluating the timewise degradation trends of biomolecules. For studies reporting p -values, we disregarded the less than signs (<) and used the absolute values, converted them to the normal deviate Z and estimated r with the following (Coltman and Slate, 2003):

$$r = \sqrt{Z^2/n}, \quad (4)$$

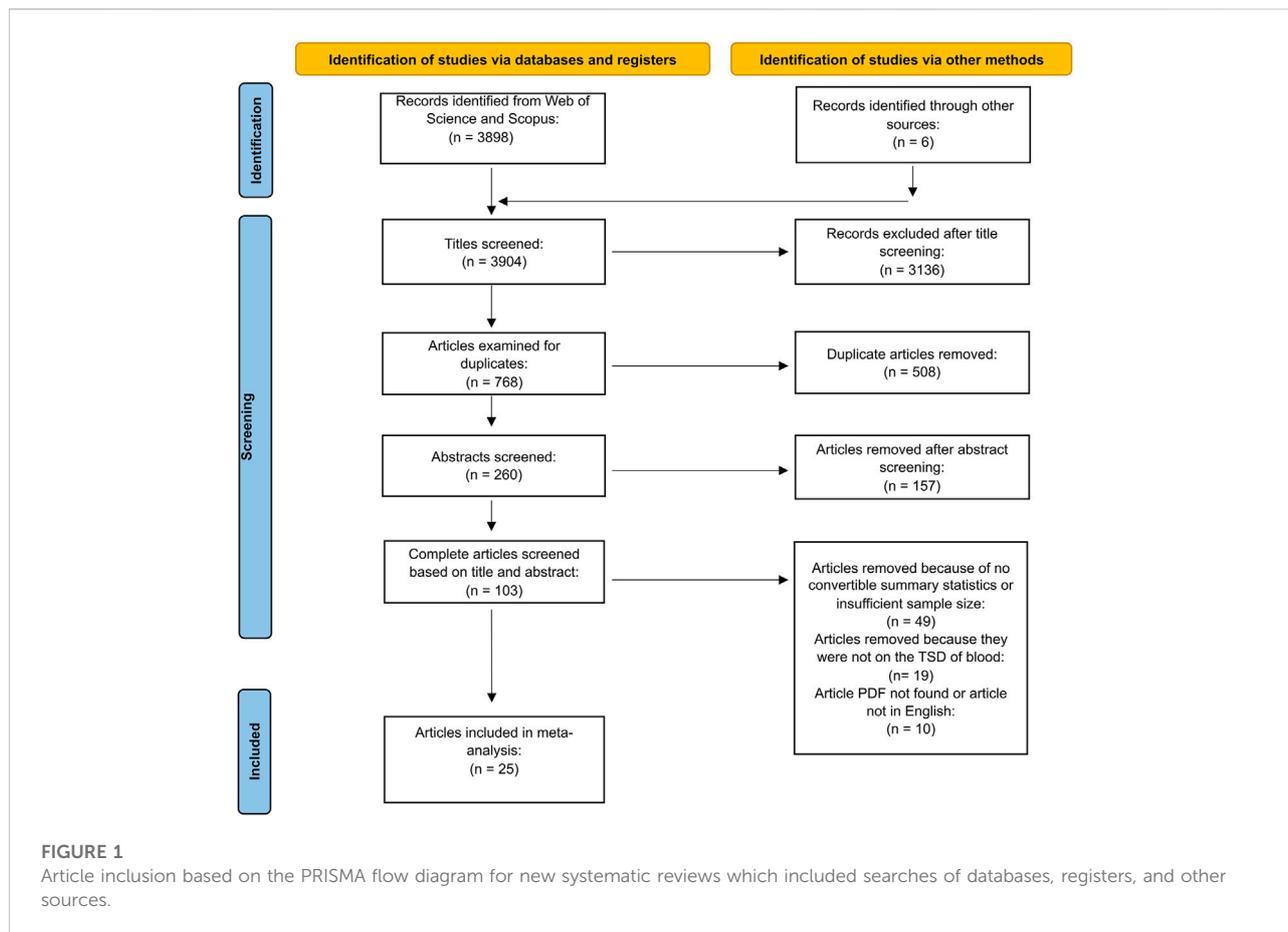
In some scenarios, values above 1 were obtained for the correlation coefficient r ; effect sizes could not be computed in those instances. For studies stating that the p -values were non-significant without reporting the numerical value, they were assigned a conservative value of 0.5 (Shafer and Wolf, 2013).

Effect sizes (Fisher’s Z_r) can range from -5 to 5 , corresponding to correlation coefficients r of -0.9999 and 0.9999 , respectively. Effect sizes closest to 0 demonstrated no correlation with time, while those furthest away in both directions represented stronger relationships (e.g. Fisher’s $Z_r = 0.5$ ($r = 0.46$), Fisher’s $Z_r = 1.5$ ($r = 0.91$)). We computed effect sizes using the absolute values of the reported summary statistic as we were interested in the magnitude and strength of the observed relationship rather than the directionality of the trend (we note that 98% were positive).

A three-level meta-analytic model was conducted using the R package *metafor* (Viechtbauer, 2010). Levels 1, 2 and 3 represent the individuals, within-study variances, and between-study variances, respectively (Cheung, 2014). Most articles included in this meta-analysis provided multiple summary statistics; a three-level model allowed for the computation of an overall mean effect size while accounting for between-study and within-study heterogeneity. We reported the overall effect size of the three-level meta-analytic model and the Higgins and Thompson’s I^2 , the amount of variation that is not attributed to sampling error, for between-study and within-study heterogeneity variances (Higgins and Thompson, 2002). An ANOVA was computed to compare two and three-level models to ensure that the model selected provided the best fit for the data. To investigate a potential publication bias, effect sizes were plotted against sample size. Egger’s regression was employed where an intercept differing from 0 in this test indicates asymmetry and possible publication bias (Egger et al., 1997).

Subgroup analyses

Subgroup analyses were conducted to assess differences in the effect sizes of the different classes of biomolecules, analytical techniques, substrate porosity, blood source, temperature and type of summary statistic reported. This was completed using three-level mixed-effects models specifying the subgroup to be evaluated (Harrer et al., 2021). Unless indicated otherwise in the



studies, ambient temperature was regarded as room temperature (18–25°C). Effect sizes and confidence intervals for each subgroup were plotted for visual comparisons; F statistics and *p*-values were recorded.

Sample size allowed us to divide our dataset by substrate porosity and construct linear mixed-effects models using data from a single porosity to explore potential drivers of Zr: we regressed Zr values against a combination of quantitative variables (e.g., blood source, temperature, and class of biomolecule) which served as fixed effects and individual studies were included as a random effect.

Results

We collected data from 33 articles where summary statistics between biomolecule degradation and TSD of bloodstains were reported; however, effect sizes were only computed from 25 articles due to insufficient sample size in eight of the retained articles (Figure 1 and Supplementary Table S2). A sample size below four cannot produce the required standard error based on Eq. 2. Most studies reported more than one

summary statistic; the 25 articles reported 109 effect sizes (Fisher's Zr) with a mean sample size of 9.7. R^2 was the most reported summary statistic, accounting for 70 of the 109, followed by correlation coefficients with 21 and *p*-values with 18. The mean effect size (Fisher's Zr) of our three-level meta-analytic model was 1.66 ($r = 0.93$, 95% CI: 0.88–0.96, p -value < 0.001; Figure 2); crudely, this means that 86% of the variation is explained by time. The estimated between-study variance was 0.74 ($I^2 = 74.8\%$), while the estimated within-study variance was 0.15 ($I^2 = 14.9\%$). We noted that the three-level model provided a better fit than the two-level model which did not account for clustering ($\chi^2 = 63.04$, p -value < 0.001, three-level: AIC = 208.4, BIC = 216.4; two-level: AIC = 269.4, BIC = 274.8).

A total of 107 effect sizes were divided into three subgroups consisting of proteins, genetic material and whole blood and cellular elements. Whole blood and cellular elements had the highest effect size and correlation with TSD, followed by genetic material and proteins (Figure 3A and Supplementary Table S3), but no significant differences were found between subgroups ($F = 0.35$, p -value = 0.71). Although whole blood and cellular elements had the largest effect size, it also demonstrated the largest

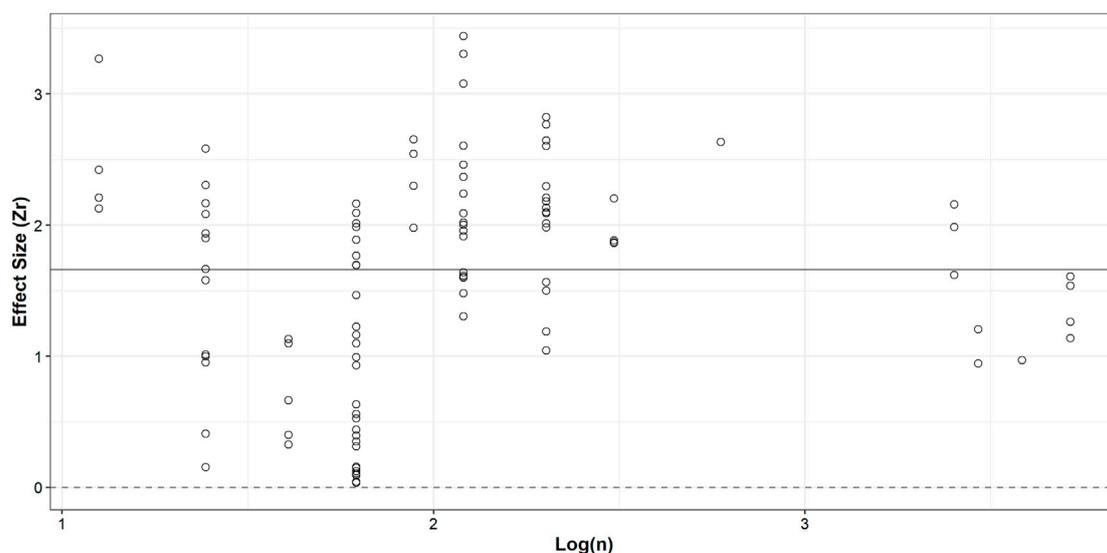


FIGURE 2 Effect sizes (Zr) plotted against log-transformed (natural logarithm) sample size, representing the number of biological replicates. The solid line at 1.66 indicates the overall mean effect size. The dashed line at 0 indicates an effect size of zero (no relationship between biomolecule degradation and TSD of bloodstains).

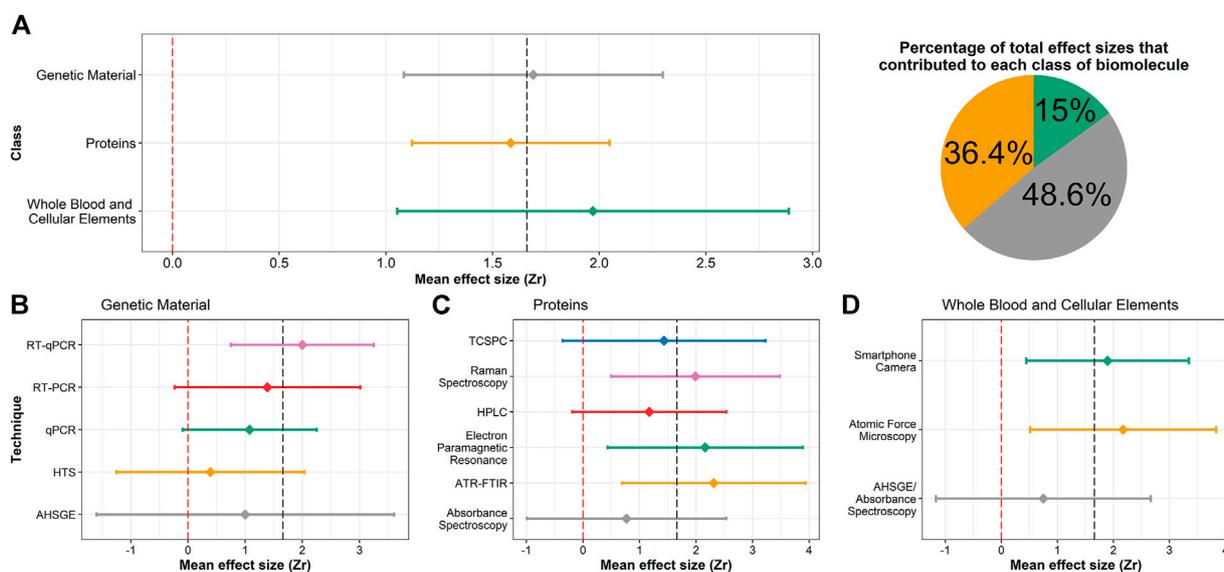
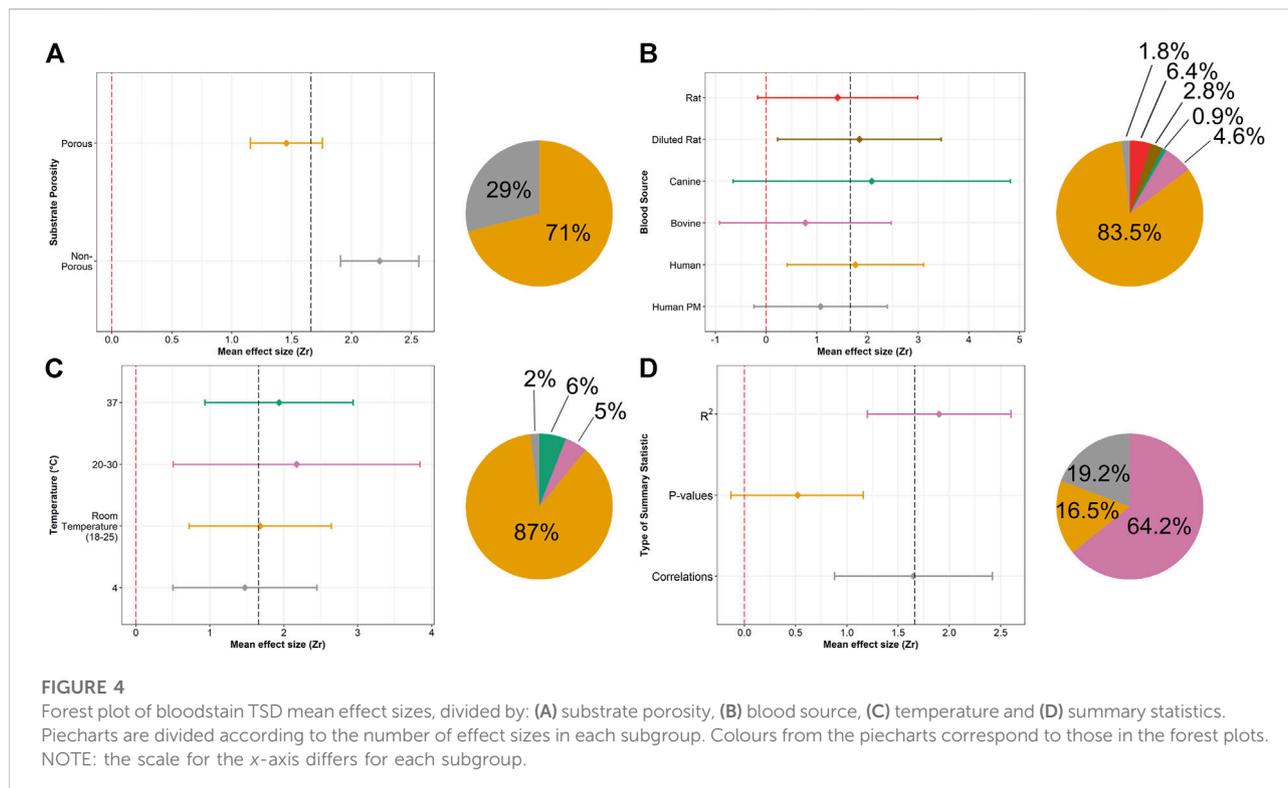


FIGURE 3 Forest plots of bloodstain TSD effect sizes for (A) class of biomolecules; (B–D) analytical techniques used in the 25 retained studies, separated by the biomolecule investigated. Diamonds indicate the mean effect size of that biomolecule or technique and the horizontal lines represent the 95% confidence interval. The red and grey dashed lines represent an effect size of 0 and the overall mean effect size of the meta-analysis, respectively. Note that the x-axis is scaled differently for (A) than (B–D). The pie chart is divided according to the number of summary statistics that contributed to each class of biomolecule; colours match those used in (A).



standard error, likely arising because of the small sample size (Figure 3A and Supplementary Table S3). For analytical techniques, 109 effect sizes were divided into 14 subgroups; mean effect sizes varied between techniques but no differences were found ($F = 1.33$, p -value = 0.21, Figures 3B–D). Most techniques exhibited broad confidence intervals because of mean effect size computation using a small number of effect sizes (Supplementary Table S3).

Effect sizes were divided into porous and non-porous substrates (Supplementary Table S3). 71% of time-series experiments were conducted using porous substrates (Figure 4A) and the effect sizes differed from non-porous substrates ($F = 22.37$, p -value < 0.001), with the latter demonstrating a greater mean effect size (Figure 4A). Blood sources were divided into six subgroups; 84% of statistics were computed from studies using human blood (Supplementary Table S3 and Figure 4B) and no difference was found between subgroups ($F = 1.15$, p -value = 0.34). Temperature was divided into four subgroups (Supplementary Table S3 and Figure 4C) and no differences were observed ($F = 0.45$, p -value = 0.72). Nearly 90% of experiments were conducted at room temperature (18–25°C). Timepoints sampled in the retained studies varied, with most studies analyzing blood after one and 2 weeks of deposition (Supplementary Figure S1). Studies that analyzed genetic material typically had longer time series than protein-based approaches (Supplementary Figure S1).

We observed minimal overlap between experimental conditions for porous and non-porous substrates, especially for the analytical techniques (Supplementary Table S4). Mixed models aimed at understanding variation in porosity Zr values showed the fixed effects for non-porous Zr often displayed significant slopes compared to porous models. Of note, conditional R^2 values were noticeably higher—approximately 35%—than the marginal values, suggesting high interstudy variation (Supplementary Table S5).

Egger's regression test produced an intercept of 2.51 (95% CI: 2.43–2.58), which was significantly different than 0 and indicative of asymmetry in our data ($t = -5.74$, p -value < 0.001), a finding consistent with the presence of a publication bias. A significant difference in reported summary statistics was found between subgroups ($F = 7.65$, p -value < 0.001). Even with the conservative approach taken to calculate effect sizes, R^2 had the greatest mean effect size of the three subgroups and was reported 64% of the time (Supplementary Table S3 and Figure 4D). p -values had the lowest mean effect size and the entire 95% confidence interval fell below the overall mean effect size (1.66).

Discussion

Our meta-analysis is the first quantitative and interdisciplinary review of bloodstain TSD and revealed that

TABLE 1 | Summary of the meta-analysis results and recommendations for future work regarding each quantitative variable.

Data type	Interpretation	Recommendation
Class of biomolecule	Large, variable effect sizes with no significant differences among studies	<ul style="list-style-type: none"> Expand biomolecule targets Consider interdisciplinary research that integrates multiple analytical techniques
Blood source	Large, variable effect sizes with no significant difference between studies	<ul style="list-style-type: none"> Use the most readily available mammalian blood source for proof-of-concept TSD studies Validate with human blood prior to crime scene implementation
Target surface	Large, variable effect size with significant difference between porous and non-porous surfaces. Greater relationship between TSD and bloodstains deposited on non-porous than porous substrates	<ul style="list-style-type: none"> Account for substrate porosity while developing experimental methodologies Focus research on non-porous substrates due to their larger effect size (i.e., appears most promising) Target non-porous substrates for the greatest probative value
Environmental	Large, variable effect size, with temperature being the only variable with sufficient summary statistics to model and analyze	<ul style="list-style-type: none"> Include additional environmental variables in TSD models (e.g., humidity, light exposure) Report exact environmental conditions for room/ambient temperature and humidity Conduct experiments at different temperatures
Analytical technique	Variable effect sizes with large confidence intervals – techniques probing the same biomolecule displayed similar effect sizes	<ul style="list-style-type: none"> Apply multiple analytical techniques where possible
Statistical Design	Variable effect sizes with significant differences between reported summary statistics – large effects sizes from R^2 and r , small effect sizes from p -values	<ul style="list-style-type: none"> Report full summary statistics Include beta coefficients (slopes) and equations for lines of best fit Provide cross-validation metrics
Publication bias	Asymmetry in data suggests publication bias, however, removal of studies with sample numbers less than 4 could indicate this is artificial	<ul style="list-style-type: none"> Publish negative results to address potential for inflation of effect size Increase sample size including number of biological replicates to quantify individual variation

there is a strong relationship between protein, genetic material, and cellular degradation in bloodstains and its TSD (Fishers $Z_r = 1.66$, $r = 0.93$). We also demonstrated that blood deposited on non-porous substrates displayed greater relationships between degradation and TSD than their porous counterparts.

Subgroup analyses

While this indicates bloodstain ageing has clear, measurable, and interpretable degradation patterns, there was a high degree of variation among studies—this is reflected by the random effect of study explaining an additional 35% on average (Supplementary Table S5). Likewise, the within-study individual variation of TSD signals can be strikingly high (Cossette et al., 2021). This leads to the TSD paradox: the overall effect time has on blood is large, but the predictive power of TSD models is small. Our meta-analysis suggests near uniformity in effect sizes related to the class of biomolecule studied, analytical technique used to measure degradation, the temperature the bloodstain was aged in, and the type of blood source used. While differences exist in the molecular properties of mammalian blood sources (Windberger et al., 2003; Kim et al., 2016; Kapusta et al., 2017; Windberger, 2019; Sparer et al., 2020; Ecker et al., 2021; Farré et al., 2021), the findings of our meta-analysis suggest that they share similar

timewise physicochemical degradation pathways of the biomolecules. This leads to a first key takeaway: most readily available mammal blood sources can be used as a human blood substitute in *proof-of-concept TSD studies*. Moreover, addition of anticoagulants allows for important experimental flexibility, mirrors human properties (Sparer et al., 2020; Orr et al., 2021) and in at least one TSD study, had minimal effect on the recorded metrics ((Elliott et al., 2022) but see (Bergmann et al., 2021)). While a variety of suitable mammal alternatives exist (Windberger et al., 2003; Ecker et al., 2021; Orr et al., 2021) and would facilitate expanded TSD research, we still recommend that for integration into forensic casework, experimentation with human blood will be required for validation.

Another noteworthy pattern is that both biomolecule and analytical techniques showed robust effect sizes, suggesting a holistic TSD approach incorporating multiple metrics is likely warranted, and in some cases, will improve model fit (Stotesbury et al., 2020), which leads to some important statistical recommendations discussed below (Table 1). The degradation of hemoglobin displayed strong timewise trends within the first weeks of deposition (Bergmann et al., 2021); the slowed rate of change once hemichromes formed limits the long-term utility of hemoglobin for bloodstain TSD estimates. mRNA and miRNA degradation appeared to be a slower process; both persisted for a long time after deposition,

sometimes up to 1 year (Weinbrecht et al., 2017; Fang et al., 2019; Salzmann et al., 2021b). A combination of spectroscopic and genomic techniques, probing hemoglobin, and nucleic acid degradation, respectively, could provide key information for bloodstain TSD research and should be combined moving forward.

Substrate porosity

We observed a difference between substrate porosity but could not attribute a specific experimental cause to the observed discrepancy (Supplementary Table S5). We suspect that the strong TSD signature observed on non-porous surfaces is linked to environmental exposure and a lack of protection from and affinity towards the surface. Marrone and Hanson (Marrone and Ballantyne, 2009; Hanson and Ballantyne, 2010) both suggested that hemoglobin oxidation occurs at faster rates in dried states compared to liquid states—since desiccation and clotting of blood take longer to occur on porous substrates than on non-porous substrates (Laber and Epstein, 2001), conversion between hemoglobin derivatives are expected to occur sooner for blood deposited on non-porous surfaces. This faster process is expected to demonstrate stronger relationships with TSD than the slower occurring processes on porous substrates, which is corroborated by the larger slopes observed for non-porous substrates in our modelling. Porous substrates might also protect the blood and biomolecules within from physicochemical degradation; greater amounts of DNA could be extracted from porous substrates than their non-porous counterparts (Verdon et al., 2011). Based on the findings of our meta-analysis, we suspect that the choice of substrate porosity is dependent in part on the proposed analytical technique, which inherently influenced experimental conditions and results. However, this leads to a second key takeaway: TSD signatures are strongest when the fluid is deposited on non-porous surfaces. To have real-world and probative value, TSD studies should focus on these types of surfaces (e.g., Doty et al., 2016; Fang et al., 2019; Cossette et al., 2021). Additionally, recently proposed relative dating approaches - in which a bloodstain from the same individual as those from the crime scene are aged under identical conditions—might mitigate many sources of variability present in absolute dating techniques and should be further explored (Menzyk, 2021).

Future directions

TSD research often has disparate experimental methodologies, particularly around sample number, type, instrumental analysis, target biomolecule, environmental ageing conditions and statistical analysis.

Despite the large effect sizes, we are far from a predictive TSD model and implementation into casework. To help address these issues, all metadata have been made available on a public

repository (<https://gitlab.com/f4301/TSD.git>). All summary statistics describing the relationship between relevant response variables and bloodstain TSD should be reported (r , R^2 , p -values, beta coefficients), and we encourage a move toward predictive modelling, cross-validation, and independent replicates. We must also increase sample sizes to fully capture variance, particularly among individuals, and report key variables like temperature and humidity values used during the study. Larger sample sizes are required for predictive modelling approaches like cross-validation. In addition, the reporting of summary statistics and use of larger sample sizes would allow for a greater percentage of studies to meet the inclusion criteria of future meta-analyses (Figure 1). Lastly, particularly regarding the development of predictive models and applications to casework, there needs to be a place for confirmatory and negative results. Our meta-analysis suggests a publication bias, not uncommon (Coltman and Slate, 2003; Shafer and Wolf, 2013; Page et al., 2021a), but in the context of developing predictive TSD models is problematic. We encourage the community to submit results to the above repository to build a database similar to DNA-Trac which documents DNA transfer studies (Gosch and Courts, 2019). Ultimately, tools are being developed to increase the precision and accuracy of bloodstain TSD estimates; these could eventually be combined with techniques estimating the time of day of deposition and the age of the donor, providing a holistic framework for all bloodstain time-related matters.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

CE: Visualization, Writing - original draft, Formal analysis, Investigation, Funding acquisition, Software, Data curation, Methodology. AS: Conceptualization, Supervision, Funding acquisition, Resources, Writing - review and editing, Project administration, Methodology, Validation. TS: Conceptualization, Supervision, Funding acquisition, Resources, Writing - review and editing, Project administration, Methodology, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/frans.2022.928573/full#supplementary-material>

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