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REVIEW

The biochemistry of the vitreous humour in estimating the post-mortem interval—a review of the literature, and use in forensic practice in Galicia (northwestern Spain)

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Abstract

The K^+ and hypoxanthine (Hx) concentrations of the vitreous humour (VH) rise gradually after death, providing a means of estimating the post-mortem interval (PMI). The correlation between these analytes and the PMI is good since the vitreous chamber is partially isolated from autolytic events occurring elsewhere; the [K +] and [Hx] recorded is thus the result of changes within the eye. The present work provides a systematic review, following PRISMA recommendations, of 36 articles (3 reviews and 33 retrospective cohort studies) discussing the many procedures and regression models that have been developed for improving PMI estimates involving VH analytes. The results of a descriptive study are also provided, highlighting the causes and distribution of mortality as registered in medico-legal autopsies performed in 2019 in Galicia (northwestern Spain), and revealing the use of these PMI estimation methods in real forensic practice. Great heterogeneity was detected in the collection of VH samples, the treatments to which they were subjected before examination, and in their conservation and analysis. A lack of reproducibility in the analytical methods employed to estimate [K +] and [Hx] was noted, as well as an absence of external validation for most of the regression formulae used to determine the PMI from analyte values. The use of methods based on high-performance liquid chromatography, focal electrophoresis, or thermogravimetric/chemometric procedures might solve the problems encountered with traditional analytical techniques, offering reliable results more quickly and effectively (even when samples are contaminated). This study recommends using flexible multiple regression models that combine physical and chemical variables, and that population databases be constructed so that models can be properly validated.

Keywords Post-mortem interval · Potassium · Hypoxanthine · Vitreous humour · Regression models · Time since death

Introduction

Estimating the post-mortem interval (PMI) has long been an important procedure in forensic investigations. Estimates can be made using a variety of methods that can be classified by reliability according to the qualitative and quantitative evidence collected [1]. In descending order, these involve:

- Collecting quantitative evidence via the use of precision tests and correcting factors; these tests are based on the

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cooling of the cadaver and the biochemistry of the vitreous humour (VH).
- Relying on subjective measurements that require the use of quantifying scales and correcting factors.
- Relying on subjective methods that involve the use of empirical estimates and correcting factors.
- Relying on subjective measurements based on empirical conclusions.
- Post-mortem changes dependent on environmental conditions.

Attempts have been made to develop methods that can be used with any type of cadaver, but to date, none has been entirely successful, a consequence of the many contextual and environmental variables to which a dead body is subject. In forensic investigations, the best PMI estimates are usually obtained by combining different methods [2], and further combining these with information provided by witnesses.

For some years, the biochemistry of the VH has received attention for estimating the PMI, with [K^+] and [hypoxanthine {Hx}] the major variables of interest. After death, VH [K^+] gradually increases [3], the result of simple diffusion across cell membranes, while VH [Hx] depends on both diffusion and the activity of the enzyme xanthine oxidase. The vitreous chamber provides protection from putrefaction for some time, offering a chance to study the post-mortem modifications it experiences independent of those affecting the external environment [4, 5].

The idea of using post-mortem changes in [K^+] as a marker of time since death was introduced by Sturner in 1963 [3]. In 2001 [6], it was established that the best way to make a regression model was by using [K^+] and/or [Hx] as the independent or predictive variable (IV). The environmental temperature is now taken into account in multivariable models that also include rectal temperature, VH [urea], and age at or cause of death [7–9].

Several functions for estimating PMI have been proposed that reflect strong correlations between the former and VH analytes. However, most have not been properly validated. This leads to results appearing better than they may really be [10].

The use of quantitative tools is uncommon. In Galicia (northwestern Spain), the PMI is frequently estimated from circumstantial evidence and cadaveric phenomena—quick and easy, but not as accurate.

This paper presents literature discussing the accuracy and reliability of estimating the PMI via the biochemistry of the VH. To determine how often VH biochemistry is used in PMI estimates made in forensic investigations in Galicia, a retrospective study was performed to examine the methods used by the *Galician Institute of Legal Medicine* (IMELGA; the public institution responsible for investigating violent and suspicious deaths in the region) to estimate PMI throughout 2019.

Material and methods

Systematic review

A systematic search was made in PubMed for papers published between 01/01/1950 and10/11/2021 focusing on VH [K^+] and/or [Hx], alone or with other variables, for estimating the PMI. This was done using MeSH terms, free terms, and combinations:

> *"Humor vítreo AND intervalo post mórtem" OR "Ciencias forenses AND humor vítreo AND potasio" OR "Humor vítreo AND PMI" OR "Humor vítreo AND (potasio OR hipoxantina) AND tiempo desde la muerte" OR "Cronodiagnóstico AND humor vítreo AND potasio" OR "Vitreous humor AND time since death" OR "Post-mortem biomarkers AND vitreous humour" OR "(Potassium OR hypoxanthine) AND vitreous humour AND time since death" OR "Thanatochemistry AND (potassium OR hypoxanthine)" OR "(Potassium OR hypoxanthine) AND vitreous humour AND regression model AND time since death" OR "Potassium AND linear correlation AND vitreous humour AND time since death" OR "PMI AND (potassium OR hypoxanthine) AND vitreous humour" OR "Vitreous potassium AND postmortem interval" OR "PMI AND thanatochemistry AND potassium AND vitreous humour" OR "Postmortem biomarkers AND (potassium OR hypoxanthine) AND vitreous humour" OR "Forensic pathology AND vitreous humour AND (potassium OR hypoxanthine)" OR "Estimation of postmortem interval AND vitreous humour" OR "Estimation of postmortem interval and* K^+*".*

The filters applied were *Full text, Classical Article, Clinical Study, Clinical Trial, Controlled Clinical Trial, Journal Article, Meta-Analysis, Observational Study, Pragmatic Clinical Trial, Preprint, Randomized Controlled Trial, Review, Systematic Review, Humans, English, Spanish, Preschool Child: 2–5 years, Child: 6–12 years, Adolescent: 13–18 years, Adult: 19 + years, Young Adult: 19–24 years, Adult: 19–44 years, Middle Aged + Aged: 45 + years, Middle Aged: 45–64 years, Aged: 65 + years, 80 and over: 80 + years, from 1950 – 2020/10/12.*

Google Scholar was also used to detect further papers in Spanish, employing search terms such as "*Humor vítreo, intervalo post mórtem, potasio, y/o, hipoxantina.* The filters used were *Desde 1950 hasta 2020, Buscar páginas solo en español.*

The resulting documents underwent further filtering by inspection of the title, abstract, and keywords. Those finally included in the analysis had to:

- Aim to determine/quantify the accuracy of PMI estimates made via VH [K^+] and/or [Hx] analysis.
- Examine correlations of the variables of interest or discuss germane adjusted regression models, providing correlation coefficients, goodness of fit, mean squared error, confidence intervals, or *p* values.
- Be either observational or experimental, a systematic review, or a meta-analysis.
- Involve a validated VH extraction technique.
- Involve cadavers not in an advanced stage of putrefaction (≤ 210 h since death), showing no large hydro/electrolyte alterations, and with death being extra-hospital in nature and of medico-legal interest.
- Involve transparent VH with no blood, and be from an intact eye of a non-decapitated cadaver.
- Involve victims over 2 years of age.
- Be written in English, Spanish, German, or French.

The references of the chosen papers were checked to detect any further works of interest missed in the above searches.

The following data were recorded from the chosen papers:

- Name of first and last author, location where study was performed, date of publication, study design, aims.
- Inclusion and exclusion criteria, sample size, extraction and treatment of VH samples, variables recorded (VH [K^+] and [Hx], other analytes, bodyweight, age, rectal temperature [Tr], among other variables.
- Results including linear determination coefficient (*R*), standard deviation, means squared error, prediction/correlation formulae, 95% confidence interval (95%CI) for PMI, and *p* value.
- Biases, methodological quality.

The criteria of the Scottish Intercollegiate Guidelines Network (SIGN) [11] were used to assess the level of evidence and degree of recommendation of the analysed reviews. Clinical trials and meta-analyses are level 1, and score ++ for little risk of bias, + for moderate risk of bias, or – for high risk. Reviews of observational studies (case control and cohort studies) are level 2, with ++ if the probability of a causal relationship is high, + if moderate, or – if poor. Case reports are level 3, and expert opinions level 4. A recommendation level A was awarded if 1 ++ or 1 + studies were applicable to their target population; B was awarded to 2 ++ studies applicable to the target population but globally consistent; C to 2 + studies applicable to the target population but globally consistent; and D to the rest.

The methodological quality of each study was decided using CASP software [12]. Reviews were given overall scores summarizing the methodological quality of the works discussed; the answers given are *yes*, *no*, or *can't tell*. Scores awarded for the results are either 1 or 0 depending on whether certain criteria are met. The review table (Table 1) constructed provides a final score for each paper of 0–10 (≥ 8 = high quality; 6–7 = moderate quality; ≤ 5 = low quality). This table also records the first and last authors, the date of publication, design, the IV and dependent variable (DV), sample characteristics, results, and observations.

Estimation of the PMI in Galicia

Information was obtained on all forensic autopsies performed in 2019 by the IMELGA centres in Galicia (those of A Coruña, Lugo, Santiago de Compostela, Ourense, Pontevedra, Ferrol, and Vigo). The data collected included age, sex, month of death, medico-legal aetiology, main cause of death, and the method used to estimate the PMI. Reports missing any of this information were excluded.

Results

Systematic review

Of the 288 articles that were initially located and processed, a final total of 36 studies were admitted for study. Figure 1 summarises the selection process.

Table 1 provides the details of the 36 studies analysed. Of these, 21 were performed in Europe, four each in the USA and India, and the remainder in Thailand, South Korea, China, Egypt, Peru, Canada, and Australia.

In cohort studies, the most frequently used analyte, alone or in combination, was VH [K^+]. In 23 studies, [K^+] or [Hx] were analysed alone or in combination with other analytes. Two studies reported the development of regression models involving VH [K^+], VH [Hx], and Tr or ambient temperature (Ta), and eight involved [K^+] and/or VH [Hx] plus VH [Na^{2+}], [Ca^{2+}], [Mg^{2+}], and [Cl^-], and the VH concentrations of urea, creatinine, albumin, uric acid, or glucose. A total of 28 studies reported only VH analytical results, one reported the same plus synovial fluid (SF) concentrations, and three examined the VH and CSF.

Two papers were narrative reviews—one a review plus a cohort study. Thirty-three studies were retrospective cohort studies. Twenty-four studies involved simple linear correlation, one involved Loess local regression, and eight involved advanced regression models, of which three combined simple and multiple linear regression.

Muñoz et al. [6] highlighted the need to swap the identity of the DV and IV. Since then, papers have identified PMI as

Table 1 Information included in the papers selected for review

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Sturner et al. [3]	Retrospective cohort study, USA	DV: [K^+], IV: PMI. Study of linear correlation coefficient between VH [K^+] and PMI	$N=91$. Samples from people who experienced sudden death outside and inside hospital but analysed separately. Cases with unknown PMI were excluded. VH was contaminated: necropsies performed > 24 h before analysis. Technical problems with some samples	Scleral puncture with 20 G, 10-mL syringe, with aspiration and freezing of samples before flame emission spectrophotometry analysis	Equation: PMI = 7.14[K^+]- 3.91 obtained a Pearson correlation coefficient of 0.987. 95%CI ± 4.7 h with a linear trend until > 100 h PMI. Up to 24 h post-mortem, predictions were more accurate	No significant differences between eyes. [K^+] higher in anterior VH. Cadavers of hospitalised people (analysed separately) returned more variable [K^+] values
Krause et al. [16]	Retrospective cohort study, Germany	DV: [K +], IV: PMI. Establishment of the correlation between VH [K +] and PMI (hours) by regression line	$N=262$ cadavers of medico-legal interest, all with known PMI	Scleral puncture with 20 G, with aspiration and storage of samples at 5–20 °C and centrifugation before flame emission spectrophotometry analysis	The correlation formula obtained was [K^+] = 2.96 + 1.65 $\sqrt{PMI(h)}$. Pearson's correlation was 0.70	There is linear correlation between [K^+] as the DV and the PMI in hours
Adjutantis et al. [17]	Retrospective cohort study, Greece	DV: [K^+], IV: PMI. Comparison of equations relating concentrations of [K^+] and PMI in using one VH sample from each cadaver in one group, and in a second group two VH samples taken at time of corpse removal from one eye, and one 3 h later from the other. Standard deviation of PMI estimates calculated	$N=120$ cadavers, all violent death-related. Sex, age, cause of death, and time since samples taken all recorded	Scleral puncture 4–5 mm from the sclerocorneal margin. 1 mL of VH taken with sterile syringe. Centrifugation: 3000 rpm for 10 min. VH [K^+] determined by flame emission spectrophotometry	Using one sample, PMI = 6.82 ± 1.36 (7.17 – [K^+]). SD PMI ± 1.7 h. Using two samples the SD was reduced to ± 1.1 h. The lower starting threshold for the increase in post-mortem VH [K^+] was 3.4 mEq/L	No difference in PMI estimates was seen with respect to sex, age, or sampled eye. [K^+] increased linearly for 12 h. Second sample allowed atypical values to be spotted if PMI > 12 h

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Aggarwal et al. [18]	Retrospective cohort study, India	DV: [K^+], IV: PMI. Establishment of the correlation between VH [K^+] and PMI (hours) by regression line	$N=99$. Samples from 50 cadavers of medico-legal interest with known PMI (12–35 h). Hospital deaths and persons with electrolytic alterations excluded. Consumption of diuretics and liquids unclear	Scleral puncture with slow aspiration; 18 G, 1.5–2-mL syringe. Samples from both eyes, analysed separately by flame emission spectrophotometry	Regression line obtained with slope of 0.52 meq/L/h. Line cuts the ordinate at 3.94 meq/L. $r=0.985$, 95%CI ± 2.26 h	No difference in PMI seen with respect to sex, age, or sampled eye. No increase in [K^+] seen after 24 h, but few cadavers surpassed that PMI, so hard to confirm
Devenaux et al. [19]	Retrospective cohort study, Belgium	DV: [K +], IV: PMI. Establishment of the correlation between VH [K +], [Na^+], [Cl^-], [Ca^{2+}], proteins, glucose, urea, and PMI (hours) by regression line	$N=37$ deaths of medico-legal interest; PMI known (10–168 h). Samples with non-transparent VH or electrolytic alterations were excluded	Scleral puncture, with slow aspiration and storage at 4 ºC. Na^+, K^+, and Cl^- were analysed with flame emission spectrophotometry, Ca^{2+} with colorimetry, glucose with glucosidase, and proteins with sulphosalicylic acid	Three linear equations were obtained: $K^+ = 0.124IPM + 7.6$ ($r=0.9$) $Na^+ = -0.261IPM + 154.9$ ($r=0.76$) $Cl^- = -0.13IPM + 128.8$ ($r=-0.43$)	There is a positive linear correlation between potassium and PMI and negative (but significant) linear correlation between sodium and chloride and PMI
Madea et al. [20]	Retrospective cohort study, Germany	DV [K^+], IV: PMI Study of differences in linear correlation coefficient in cadavers with electrolyte imbalance and/or > 6 h of death agony	$N=70$ deaths of medico-legal interest separated by time of agony (< 6 h/ > 6 h) and [Urea] in VH (< 100 mg/dL/ > 100 mg/dL)	Scleral puncture with 20 G, 10-mL syringe, and storage at − 70 °C. Centrifugation at 3500 rpm 7 min after flame emission spectrophotometry analysis	Equation: $PMI = 0.187[K^+] + 6.1$ obtained a Pearson correlation (r) coefficient of 0.843. In the [Urea] < 100 mg/dL r was 0.926 and in the < 6 h of agony was 0.953	Pearson's correlation was higher in cadavers with no electrolyte imbalance and < 6 h of death agony

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Stephens et al. [21]	Retrospective cohort study, USA	DV: [K^+], IV: PMI. To study a large sample to see if a good linear correlation exists between VH [K^+] and PMI	$N = 1427$. Deaths of medico-legal interest; PMI known (0–35 h). Samples with values > 2 SD of the mean excluded. Sudden death of nursing baby, and persons with non-transparent VH, electrolytic alterations, who were intoxicated, who had hypo/hyperthermia, or who drowned were also excluded	Scleral puncture with 18 G, 5–30 mL syringe, centrifugation at 3000 rpm for 10 min, and storage at –20 °C. Supernatant analysed by indirect potentiometry	PMI = 0.258[K^+] + 6.342, R 0.374, 95%CI ± 20 h. Conclusions not extrapolated beyond 35 h PMI	62.3% of the variance in [K^+] inexplicable by PMI. 95%CI was narrow due to the taking of a single sample from each cadaver. Would have been more informative to study the linear coefficient determination (*R*)
Schmidt et al. [22]	Retrospective cohort study, Germany	DV: [K +], IV: PMI. To study if scleral punctures of both eyes obtained in different times have different slopes in the linear regression model	$N = 53$ cadavers of medico-legal interest, all with known PMI (max 90 h)	Scleral puncture with 20 G, 10-mL syringe, with aspiration and storage at –70 °C and centrifugation after flame emission spectrophotometry analysis	These samples of both eyes obtained < 4 h from each other got a linear slope of 0.18 and a Pearson coefficient of 0.77, while these obtained > 4 h got a slope of 0.19 and a coefficient of 0.80	There are no significant differences between the correlation and regression slopes of samples obtained of both eyes in less than 4 h or more than 4 h
Coe et al. [23]	Narrative review, Ireland	DV: [K^+], IV: PMI. To examine the different studies that used VH [K^+] to determine PMI to see if a correlation exists (regression analysis), and to examine the accuracy of PMI estimates (confidence interval analysis). Studies the factors that might modify the accuracy of PMI estimates	Included human and animal studies that examined VH [K^+] in recent cadavers. Studies published in English	Scleral puncture with complete aspiration using 20 G, 10-mL syringe. Analysis by flame emission spectrophotometry and indirect and direct potentiometry	Linearity between [K^+] and PMI, but with large variations between studies. SD as large as ± 26 h. Regression slopes from 0.14 to 0.55 mEq/L/h). The formula of Sturner et al. [3] in which PMI = 7.14[K^+]—3.91 was the most accurate with errors of up to 4.7 h (95%CI)	External factors and sampling method (scleral puncture done slowly/more quickly and removing all/not all the VH possible, etc.), and treatment of the sample, all found to influence the results. Temperature increased VH [K^+] significantly above 10 °C—more so in children than in adults (specific graphs needed). Urea up to > 100 mg/dL also related to [K^+]

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Madea et al. [24]	Retrospective cohort study, Germany	DV: $[K^+]$, $[Na^+]$, $[Cl^-]$, [urea] and $[Ca^{+2}]$, IV: PMI. To measure the accuracy of, and adjust, a regression line for these VH analytes, and to take into account the influence of VH [urea] as a marker of electrolyte disequilibrium	$N=170$ (100 sudden deaths and 70 due to chronic disease). Performed in three steps: with all samples, with all excluding those with VH [urea] > 100 mg/dL and excluding those with an associated agonal period of > 6 h	Scleral puncture with complete aspiration using a 20 G, 10-mL syringe, centrifugation (3000 rpm, 10 min), and analysis of supernatant for $[K^+]$, $[Na^+]$, and $[Cl^-]$ by indirect potentiometry, of [urea] by enzymatic methods, and of $[Ca_2^+]$ by colorimetric techniques	95%CI for $[K^+]$ for whole sample set ± 34 h ($r=0.86$). After excluding samples with [urea] > 100 mg/dL this fell to ± 22 h ($r=0.89$), and when excluding samples associated with an agonal period of > 6 h, it fell to ± 20 h ($r=0.91$). The respective regression slopes were 0.203, 0.187, and 0.186	This study revealed significant differences in analyte concentrations between eyes during the same period. The best analyte for determining PMI was $[K^+]$, and [urea] the best for assessing electrolyte disequilibrium
Rognum et al. [25]	Retrospective cohort study, Norway	DV: $[K^+]$ and [Hx], IV: PMI. To study the correlation between the aforesaid analytes and their variation as influenced by Ta	$N=87$ cadavers of medico-legal interest, all with known PMI and classified according to Ta (5 °C, 10 °C, 15 °C, and 23 °C. Contaminated samples were excluded, as were those taken from trauma-damaged eyes	Scleral puncture with complete aspiration. Analyses performed by HPLC for [Hx], and flame emission spectrophotometry for $[K^+]$	Slopes of 0.17, 0.2; 0.25 and 0.3 were obtained respectively for the four Ta groups for $[K^+]$, and of 4.5, 5.1, 6.2, and 8.8 for [Hx]. The regression line intercepted the ordinate at 7.60 for [Hx] and 5.8 for $[K^+]$. $p<0.001$. $r=0.93$ for both analytes	The diffusion of K^+ into the VH lasted longer than the appearance of Hx. Thus, the latter is best used with cadavers < 24 h old (in which $[K^+]$ is more variable) with no pre mortem hypoxia. VH $[K^+]$ was related to VH [urea]
Gamero et al. [26]	Retrospective cohort study, Spain	DV: $[K^+]$, IV: PMI. To study and compare, using the formulae proposed by 11 sets of authors, the correlation between VH $[K^+]$ and PMI via linear regression	$N=76$, i.e., 56 in-house and 20 from the work of Umani et al. [21]), all taken from cadavers of medico-legal interest and with a PMI of < 24 h	Scleral puncture with complete aspiration using a 20 G, 5-mL syringe Samples were stored at − 70 °C, centrifuged and analysed for $[K^+]$ by flame emission spectrophotometry	The best regression lines were (in descending order) those of Gamero et al. [20], Cespedes et al. [22], Madea et al. [18], and Adjutantis et al. [14]. The formulas of Cespedes et al. [22] and Adjutantis et al. [14] underestimated the PMI, while that of Sturner et al. [3] overestimated it. The 95%CI for m was 0.0902–0.2268, and for n 5.2585–7.437	The samples returned no fixed value for the PMI. This prevented homoscedasticity checks. Thus, the results could not be extrapolated to cadavers of > 24 h

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Lange et al. [15]	Narrative review and literature re-analysis, USA	IV: [K^+], DV: PMI. To study the non-linear correlation between [K^+] and PMI via non-linear regression, employing the Loess local regression model	$N=790$ cases reviewed from six studies dated 1963–1980 all of which used linear regression to estimate the PMI	With the permission of the different study authors, all cases were reviewed under the assumption of there being a non-linear correlation between [K^+] and PMI	Simple linear regression involving all the data returned $K^+ = 0.173\ PMI + 5.69$, with $R = 0.72$. The examined relationship was concluded to be non-linear (even when re-scaling with square roots) Local regression confirmed a non-linear relationship. 95%CI varied depending on K^+: t K^+: < 7 mEq/L ± 1 h, 7–12 mEq/L ± 2 h, 12–18 mEq /L ± 3 h, > 18 mEq/L ± 5 h	The PMI was (in novel fashion) understood to be the DV. The Jacknife tool was used to determine whether the Loess local regression predictions were appropriate for examining new data
Madea et al. [27]	Retrospective cohort study, and narrative review, Germany	DV: [K^+] and [Hx], IV: PMI. To examine [K^+] and [Hx] as predictors of PMI, and to determine the validity of [Hx] as a marker of hypoxia in the VH and CSF	$N=92$ samples from cadavers of medico-legal interest, with a PMI of 2–20 h. The cadavers of persons with chronic disease or that provided contaminated samples were excluded. Samples were taken from both eyes	Scleral puncture of both eyes at the same time. Samples also taken from the cisterna magna. [K^+] determined by indirect potentiometry, and [Hx] by HPLC	The [Hx] was more sensitive at very early PMIs. At other times [K^+] was more accurate ($r=0.925$) than [Hx] (0.715) (95%CI ± 17 h for [K^+] and ± 32 h for [Hx]). The variation in [Hx] as a marker of hypoxia in different studies, and its difficult analytical management, leave it in disadvantage with respect to [K^+] for estimating the PMI	VH [Hx] increased immediately after death up to 48–72 h post-mortem. Differences between the eyes in terms of [Hx] were (unexpectedly) smaller when the PMI was longer. In the CSF the increase was not linear. Newborns showed higher concentrations of both analytes. Although hypoxia did not affect them significantly, if only mild to moderate it did influence the result since the period of relative hypoxia was longer

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Muñoz et al. [6]	Retrospective cohort study, Spain	DV: PMI, IV: [K^+]. To determine the linear regression equation with [K^+] as the IV, and to study the fit and accuracy of the model in estimating PMI	$N=201$ samples from cadavers of medico-legal interest, all with known PMI. Sex, age, cause of death, time to extraction of the VH, and results of toxicological analyses all known. Non-transparent samples were excluded. Only samples from cadavers with a PMI known within a ± 15 min window were used	Samples taken by scleral puncture using a 20 G, 10-mL syringe, followed by centrifugation (3000 rpm, 10 min) Analysis by indirect electrode potentiometry, and by time extraction analysis within 24 h (stored at 4 °C until then)	PMI = 3.92[K^+]-19.04 for non-hospital deaths. R 0.70. When [K^+] 8–12 mEq/L, error in PMI = ± 1.29 h (accuracy greatest in this range)	Excluding samples with VH [urea] < 30 mg/dL and [creatinine] ≥ 0.5 mg/dL improved the accuracy of PMI estimates. Using [K^+] as the IV improved the variance explained by the model (0.7 compared to 0.57 for traditional methods), reducing the error
Muñoz et al. [9]	Retrospective cohort study, Spain	DV: PMI, IV: [K^+] and [Hx]. To establish a regression formula correlating PMI with VH [K^+] and [Hx], and to check for variations according to cause of death	$N=206$ samples from cadavers of medico-legal interest (2 groups: hanged and not hanged) with known PMI (error ± 15 min). Non-transparent samples excluded. Cadavers of children under < 6 months, and samples with [urea] < 30 mg/dL also excluded	Extraction by scleral puncture; samples analysed within 48 h (VH centrifuged). [K^+] determined by indirect electrode potentiometry. Samples stored at 4 °C	[K^+] was 72% greater, and [Hx] 55% greater, in samples from hanged cadavers. Adjusted formulae obtained were: PMI = 3.631[K^+] – 17.344 ($R=0.688$). PMI = 0.153[Hx] – 0.368 ($R=0.518$). Goodness of fit tested using by Durbin Watson statistic (1.73 for [K^+] and 1.575 for [Hx]) and the Cook distance (0.098 for [K^+] and 0.145 for [Hx])	Accuracy was greater for the hanged cadavers (23% higher for [K^+] and 55% for [Hx]). It improved further when VH [urea] > 30 mg/dL The increase in cranial venous pressure during hanging would cause more of these analytes to accumulate in the VH
Madea [2]	Narrative review, Germany	To review the state of the art in estimating MPI via VH biochemistry (among other techniques)	Autolysis occurs in the blood in the first hours after death, in the CSF at 15–20 h, and in the VH after 120 h. The regression slope increases over time. In cadavers with > 100 mg/dL, the 95%CI was better (± 20 h). [K^+] was found to be a good marker of electrolyte alterations. Using PMI as the DV reduced the error of estimates somewhat (from ± 25.96 h when the PMI was the IV, to ± 23.27 h). The relationship between [K^+] and PMI was deemed not to be entirely linear. The residual variability was not constant. A non-linear approximation was therefore thought to be better (as long as [K^+] not very high)—although this was not confirmed in different populations. Multiple regression involving many VH analytes improved accuracy of PMI estimates (95%CI ± 16.2 h). Capillary zone electrophoresis with 50 qL samples was more accurate than traditional methods, and pre-treatment with heat (100 °C, 5 min), centrifugation, and digestion with hyaluronidase was deemed acceptable (causing no differences), although more studies are needed to determine the gold standard. [Hx] was also found to be a good marker of PMI (less dispersion in concentration was seen than for [K^+]), although [K^+] provided the best estimates			

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Mulla et al. [28]	Retrospective cohort study, Canada	DV: PMI, IV: [K^+], [Cl-], [Na+], and [Ca^2+]. To study the correlation between the two eyes with respect to the above analytes, and to see how differences might affect estimates of PMI	$N=48$ samples form cadavers of medico-legal interest, all with known PMI (4.5–84.3 h). Samples taken from both eyes for immediate analysis. Samples from cadavers belonging to people with chronic disease were excluded, as were those taken from trauma-damaged eyes	Scleral puncture with complete aspiration of (both eyes) using 20 G, 12-mL syringe, followed by centrifugation (2050 rpm, 10 min). Supernatant analysed immediately by indirect potentiometry	Linear adjustment of [K^+] with PMI gave an R value 0.739). Only 8% of cadavers returned > 20% different results for the two eyes—these differences did not modify the PMI estimate The correlation between PMI and [K^+], [Cl-], and [Ca^{2+}] was $R=0.948$, 0.492, and 0.469 respectively	The largest variations between eyes were seen in terms of VH [Na^+] and [Cl-] (12 and 8 meq/L respectively). Differences were seen between the eyes when measuring by potentiometry, but not by flame emission spectrophotometry
Madea et al. [29]	Retrospective cohort study, and descriptive study (re-evaluation), Germany	DV: PMI, IV: [K^+] and [Hx]. To analyse the accuracy of estimates of PMI using VH [K^+] and [Hx] as IVs. A re-evaluation of the statistical results offered by Lange et al. [15] is offered	N1 = 544 (170 in-house samples analysed for [K^+], and 198 s analysed for [Hx], plus 176 from the study of Muñoz et al. [4], and) N2 = 523 cadavers with known PMI (error ± 15 min) for re-evaluating the study by Lange et al. [15]. Samples that were non-transparent, from cadavers belonging to people with electrolyte alterations, chronic diseases, or hypo/hyperthermia, were excluded	Samples taken by scleral puncture with complete aspiration using 20 G, 10-mL syringe, followed by centrifugation (3000 rpm, 10 min). Analysis for [K^+] and [Na+] by indirect electrode potentiometry, and for [Hx] by LC-MSMS. Samples were stored at − 70 °C	The use of [K^+] and [Hx] as VIs increased the accuracy of PMI estimates: ± 23.27 h (± 7.15 h using samples from Muñoz et al. [6]; and ± 13.69 h with [Hx]). $R=0.742$. The PMI was overestimated by Lange et al. [17] by some 6 h (SD ± 12 h)	Significant increase in accuracy obtained when considering the analytes as IVs (as determined by Pitman T tests). Loess local regression analysis of the results of Lange et al. [15], and the data of Muñoz et al. [6], showed the latter authors to estimate PMI more accurately

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Zhou et al. [13]	Retrospective cohort study, China	DV: [K^+], IV: PMI. To assess the linear correlation between VH [K^+] and PMI	N = 62 samples form cadavers of medico-legal interest, all with known PMI (1–27 h). Cadavers of people showing signs of trauma or who had eye disease were excluded, as were those with traumatic brain injury, and severe electrolyte alteration. Putrefied cadavers were also excluded	Microscleral puncture (100 µL). Analysed samples of 4 µL; rest stored at 4 °C). Analysis by low-pressure ion chromatography	The equation [K^+] = 0.1702 PMI + 5.5678 returned an *r* value of 0.8692; the technique is therefore valid for examining VH biochemistry	The technique is a simple, rapid, and cheap way of testing VH biochemistry
Bortolotti et al. [14]	Retrospective cohort study, USA and Italy	DV: [K^+], IV: PMI. To study the correlation between VH [K^+] and PMI	*N* = 164 samples from cadavers of medico-legal interest, all with known PMI (1.8 h a 109 h). All were aged between 11 and 88 years. Cadavers of people with chronic disease, eye trauma, and chronic kidney disease or who were dehydrated were excluded	Microsamples (100 µL) taken with 25 G syringe with aspiration of the ocular posterior chamber. Samples stored in plastic vials at − 20 °C. [K^+] analysed by capillary electrophoresis	The formula [K^+] = 0.1733 (PMI) + 2.3, returned $R = 0.962$ (SD ± 5.54 h). With specific formulae for cadavers < 24 h and > 24 h, R values of 0.709 and 0.955 were respectively obtained	Interindividual variation was significant until 24 h, when [K^+] was still little concentrated. After this time the differences were not significant. [K^+] was found to be a good marker of PMI until 100 h post-mortem
Chandrakanth et al. [30]	Retrospective cohort study, India	IV: [K^+], [Na+], [Cl-], [Na+]/[K^+]. DV: PMI. To determine whether a correlation exists between these analytes and PMI, and to examine the differences between eyes and between sexes	N = 114 samples from cadavers of medico-legal interest, all with known PMI (0–36 h) Cadavers of people with chronic disease, eye trauma, traumatic brain injury, blood-contaminated VH, were excluded. No pre-death analytical data were available	Scleral puncture with complete aspiration of both eyes, using 20 G, 10-mL syringe, followed by centrifugation (4500 rpm, 10 min). Supernatant immediately analysed by indirect potentiometry	No significant differences in concentrations between eyes, or between men and women. Correlation coefficients for PMI with [Na+], [K^+], [Cl-], and [Na+]/[K^+] were − 0.025, − 0.039, − 0.047, and 0.057 respectively (not significant)	Groups were made for PMI 0–6 h, > 6–12 h, > 12–18 h, and > 18 h). [Na+] and [Cl-] increased in men, but not significantly. As PMI increased, the [Cl-] and [Na+] fell while [other analytes] increased [Cl-], [Na+] and the creatinine, lactate and urea concentrations were more stable

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Mihailovic et al. [31]	Retrospective cohort study, Serbia	DV: PMI, IV: $[K^+]$. To determine whether repetitive extractions of small VH volumes improved the accuracy of PMI estimates	$N=320$ samples from cadavers of medico-legal interest (ages 18–65 years), all with known PMI (3–30 h). Cadavers of people who had been hospitalised, had chronic disease, sepsis, kidney disease, were intoxicated by drugs, or had high VH [urea] concentrations, were excluded	Repetitive punctures, with 0.1 mL 20 G syringe, then 2 mL, followed by centrifugation (16,000 rpm, 10 min). Supernatant analysed for $[K^+]$ by indirect potentiometry. Samples stored at − 20 ºC	The equation PMI = 2.749(K^+)—11.978 returned r = 0.923 (95%CI ± 2.8 h). The differences between the results obtained and those expected varied by 2 ± 2.3 h in cadavers at 20 °C, and by 2.3 ± 2.2 h in those at 4 °C	The small scaled repetitive punctures had no effect on $[K^+]$ in the late phases of extraction. Little variation has been seen between the obtained and expected results
Nermeen et al. [32]	Retrospective cohort study, Egypt	IV: $[K^+]$, DV: PMI. To study the linear relationship between VH $[K^+]$ and PMI, and to see if there are differences between eyes and sexes. The influence of Ta was also examined	$N=120$ samples from cadavers of medico-legal interest, all with known PMI (0–100 h) Cadavers of people with eye disease, eye trauma, chronic kidney disease, or who were dehydrated, were excluded	Scleral puncture using 20 G, 1 mL syringe, and aspiration (both eyes), followed by centrifugation (3000 rpm, 10 min), and storage of supernatant at − 60 °C. $[K^+]$ analysed by flame emission spectrophotometry	The formula PMI = 8.77 K^+—53.90 retuned a Pearson's correlation coefficient of 0.7918 ($p<0.05$, 95%CI ± 14.44 h). Linear correlation shown between $[K^+]$ and PMI; best results after 24 h PMI	No significant variation in $[K^+]$ between eyes. Ta seemed to have no influence on estimates
Tumram et al. [33]	Retrospective cohort study, India	DV: PMI, IV: $[K^+]$. To study the linear relationship between VH $[K^+]$ and PMI by linear regression, and to compare the line obtained with others reported in the literature	$N=308$ samples from cadavers of medico-legal interest, all with known PMI (1.45–35.18 h). All were of known age and sex. The cause and circumstances of death were also known. Non-transparent VH samples were excluded	Scleral puncture using a 20 G, 1.5–2 mL syringe, followed by centrifugation (3500 rpm, 10 min). Immediate analysis of supernatant by indirect potentiometry	The formula PMI = 2.71$[K^+]$—20.19 returned a correlation coefficient of 0.526 and 95%CI of ± 5.8 h. Regression slope: 0.36 mmol/L/h. Line bisected ordinate 7.43 mmol/L	Comparison of 7 published studies. Regression line bisected the ordinate at a similar value for all, although the slope was steep for the Indian data due to the high Ta recorded. The slope was steeper in the first 6 h PMI, with stronger correlation seen after 12 h

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Swain et al. [34]	Retrospective cohort study, India	DV: PMI, IV: [K^+], [Na+], [glucose], [Ca^{2+}], [creatinine], and [urea]. To determine whether a correlation exists between VH and CSF analytes, and to determine the best variable for estimating the PMI	$N=100$ samples from cadavers of medico-legal interest with PMI < 96 h. Cadavers of people with meningitis, encephalitis, septic shock, eye trauma, metabolic syndrome, and intracranial haemorrhage were excluded	Scleral puncture using a 16 G, 2 mL syringe, followed by centrifugation (3000 rpm, 10 min). Samples stored between − 18 and − 70 °C. [K^+] and [Na+] determined by indirect potentiometry; glucose, creatinine, and urea enzymatically; [Ca^{2+}] by chromogenic methods	[K^+], [Na+] and [glucose] correlated in both fluids ($r=0.62$–0.63 and -0.27 in VH, and 0.37–0.35 and 0.34 in CSF respectively). Only [K^+] and [glucose] showed a significant difference in r as PMI increased	No significant difference between VH and CSF analytes, or in men compared to women. The best variable was [K^+]. VH performed better than CSF, except for [Na+]
Rognum et al. [35]	Retrospective cohort study, Norway	IV: PMI and Ta DV: [K+], and ([K+] *Ta. The paper is focused on the use of regression analysis of the vitreous humour (Hx) and (K^+) as well as the ambient temperature to calculate an algorithm for the estimation of PMI	$N=132$ samples from cadavers of medico-legal interest, all with known PMI (< 60 h), distributed in 5 groups according to Ta (5 °C, 5.1–10 °C, 10.1–15 °C, 10.1–20 °C, and > 20 °C). [Hx] was measured in all, and [K^+] in 106	Scleral puncture with complete aspiration using a 20 G, 10 mL syringe, followed by centrifugation (9000 rpm, 90 min). Supernatant stored at − 20 to − 75 °C. [Hx] determined by HPLC, and [K^+] by flame emission spectrophotometry	The formulae PMI = 0.215[Hx]—0.467Ta 0.005[Hx]Ta + 10.353 and PMI = 5.164[K^+]—0.174Ta-0.1[K^+] Ta -19.58 returned *R* values depending on Ta group. For [Hx]: 0.76, 0.76, 0.86, and 0.88 respectively; for [K^+]: 0.77, 0.78, 0.57, and 0.9. For non-linear multivariate models that combined [Hx] and [K^+], the goodness of fit was 0.89 with a standard error of ± 2.5 h for [Hx] and ± 3 h for [K^+] +	R increased with the age of the cadaver. However, given the characteristics of the aged cadavers sampled, no conclusions were drawn regarding whether such age had a significant effect on the PMI estimate or *R* value. Sex was not found to significantly modify estimates of PMI The use of diuretics, alcoholism, and chronic kidney disease all modified the VH [K^+]

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Ortmann et al. [36]	Retrospective cohort study, Germany	DV: PMI, IV: [K^+]. To study the linear relationship between VH [K^+] and PMI in European cadavers via the comparison of the linear regression formulae provided in 5 studies	$N=600$ samples from cadavers of medico-legal interest, all with known PMI (error ± 15 min). Non-transparent samples, and samples from cadavers of people with chronic disease or large electrolyte alterations were excluded	Scleral puncture with complete aspiration of both eyes. Samples analysed by indirect potentiometry at different laboratories. Samples were grouped with respect to [K^+] (5–10 mmol/L, 10–15 mmol/L, or > 15 mmol/L), and according to PMI (< 30 h, 30–60 h and > 60 h)	For Central Europe, lines with slopes of 0.17–0.19 mmol/L/h and crossing the ordinate at 5.85 mmol/L showed more homogeneous correlation values without large variations. The models of Madea et al. [27] and Muñoz et al. [6] (95%CI ± 20 h) were the most accurate. In warmer climates, the regression slope is steeper	The line of Sturner et al. [3] overestimated PMI (less steep slope), while that of Jashnani et al. [37] overestimated it (the slope was steeper given the hotter climate in this study). The standard deviation between the observed and expected results increased with PMI and VH [K^+]
Tatiya et al. [38]	Retrospective cohort study, India	DV: PMI, IV: [K^+]. To study the correlation between VH [K^+] and PMI, and the time window over which [K^+] increases	$N=207$ samples from cadavers of medico-legal interest, all with known PMI. Cadavers of people with chronic disease, eye trauma, who were hospitalised, took diuretics, who had large electrolyte alterations, or whose PMI window was over ± 15 min, were excluded	Scleral puncture using a 21 G, 10 mL syringe and complete, slow aspiration, followed by centrifugation (3000 rpm, 10 min) and analysis of the supernatant by flame emission spectrophotometry	Linear correlation detected. The formula PMI = 0.204[K^+] + 9.6315 returned an r value of 0.6694 and a 95%CI of ± 16.74 h. VH [K^+] increased constantly from 0–46 h post-mortem	Most reported studies indicate VH [K^+] to rise over a longer period than 46 h. The discrepancy might be explained by the high temperatures encountered in India causing equilibrium to be reached more quickly. Scleral puncture recommended to be slow and complete

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Cordeiro et al. [7]	Retrospective cohort study, Spain and Portugal	DV: PMI, IV: [K^+], [Hx], Ta, Tr, bodyweight and [urea] combined in different formulae. To establish a formula taking into account VH analytes and physical factors, to generate the best fitting regression possible, and to determine which variables most influence PMI estimates	$N=331$ samples from cadavers of medico-legal interest, all with known PMI (error ± 15 min) Non-transparent samples, and samples from cadavers of people with chronic disease or large electrolyte alterations, or who suffered hyper/hypothermia, were excluded. The sex, age, bodyweight, Tr, Ta at body removal, cause of death, and attempts to resuscitate were known	Samples taken by scleral puncture (both eyes) with complete aspiration using a 20 G 10 mL followed by centrifugation (3000 rpm, 10 min). [K^+] and [Na+] determined by indirect electrode potentiometry; urea uric acid, xanthine, [Hx] and [glucose] by LC-MSMS. Time to analysis following extraction under 24 h. Storage at 4 °C. Mean results for both eyes together were recorded	Five methods established for estimating PMI. The highest R value (0.786; mean squared error 0.077) was obtained with: $\log(\mathrm{IMP}) = \alpha + f_1(\mathrm{Tr}) + f_2(\mathrm{Ta}) + f_3(\mathrm{K}^+) + f_4(\mathrm{Hx}) + f_5(\mathrm{urea}) + f_6(\mathrm{peso}) + \varepsilon$. That which minimised the mean squared error (0.074; $R=0.784$) and provided the best predictions was $\log(\mathrm{IMP}) = \alpha + f_1(\mathrm{Tr}) + f_3(\mathrm{K}^+) + f_4(\mathrm{Hx}) + f_5(\mathrm{Urea}) + f_6(\mathrm{peso}) + \varepsilon$ It was recommended the variable shown in these two equations be used	Values were log-transformed to ensure homoscedasticity and symmetry. The cadaver storage temperature and sample age influenced the PMI estimate, but not significantly so. The cause of death, resuscitation manoeuvres, age, sampled eye (right or left), and sex had no influence either This is the only paper examined in this review with an externally validated model
Srettabunjong et al. [39]	Retrospective cohort study, Thailand	DV: PMI, IV: [K^+], [Na+], glucose, lactate and urea, uric acid, and creatinine concentrations, [Cl-] and [Mg2+]. To determine whether a correlation exists between these analytes in VH and SF, and which are best for calculating the PMI in recent cadavers (< 8 h)	$N=35$ samples from cadavers of medico-legal interest, all with known PMI (< 8 h), and with transparent VH and SF Samples from cadavers of people large hydro/electrolyte alterations, with chronic kidney disease, that were submerged in water, died in surgery, or who had eye-area trauma were excluded Age, sex, bodyweight, height Ta, circumstances surrounding death, and cause of death were known	Scleral puncture subpatelar samples taken with complete aspiration using a 20 G, 5 mL syringe followed by centrifugation (13,000 rpm, 10 min) and storage of the supernatant at − 80 °C. [K^+], [Na+], and [Cl-] determined by indirect electrode potentiometry; [Mg2+] by chromogenic methods; glucose, urea, uric acid, creatinine, and lactate by enzymatic methods	VH [K^+], [Na+], [Cl-], and [Mg2+] were higher than in the SF, while glucose, creatinine, and uric acid were higher in SF than in VH. No differences in urea or lactate. Significant correlation between VH and SF [K^+], [Na+], [glucose], [creatinine] [urea], [lactate], and [uric acid], but not [Cl-] or [Mg2+]. Both VH and SF analytes deemed to be useful in determining PMI	Strongest correlations seen for urea and creatinine ($R=0.987$ and 0.899 respectively) when using the formulae: [urea] VH = 2.903 + 0.825[Urea] SF and [Cr] VH = − 0.172 + 0.712[Cr] SF. SF could be used to determined PMI

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Go et al. [40]	Retrospective cohort study, South Korea	DV: PMI, IV: lactic acid and [Hx]. To determine whether lactic acid and [Hx] are good predictors of PMI corrected by season of year and Ta	$N=79$ samples form cadavers of medico-legal interest, all with known PMI (13–103 h). Lactic acid and Hx measured in both eyes. Samples grouped by Ta and season Samples from cadavers of people with large hydro/electrolyte alterations, with eye trauma, or with kidney disease, were excluded	Scleral puncture with complete aspiration using a 20 G, 10 mL syringe, followed by centrifugation (3000 rpm, 10 min). Hx and lactic acid in supernatant determined by LC–MS/MS. Samples stored at − 20 °C	The R value for [Hx] was 0.53, and 0.59 after correction for Ta. The R values for the seasons were 0.43. 0.58, 0.49, and 0.8 for spring, summer, autumn, and winter, respectively. For lactic acid, R was 0.38, rising to 0.42 after correction for Ta, and 0.22, 0.28, 0.4, and 0.71 after correction for the different seasons. For [Hx], adjusting for Ta, the R values were 0.84, 0,69, 0.88, and 0.81, while for lactic acid they were 0.42, 0.29; 0.48, and 0.72	The correlation between PMI and both lactic acid and [Hx] became poorer as the temperature increased. In winter R was 0.8 and in summer 0.43 for [Hx]. The correlation did not vary significantly for small changes in Ta summer or winter but did so in autumn and spring with their large changes in temperature
Saldaña et al. [41]	Retrospective cohort study, Peru	IV: [K^+], [Hx], [urea], DV: PMI. To determine whether a correlation exists between PMI and these variables via multiple regression	$N=73$ samples from cadavers of medico-legal interest. Only 35 were not excluded—the rest were rejected because of eye trauma or eye disease, unknown PMI, or non-transparent VH. Sex, age, and cause of death were known	Scleral puncture using a 20 G, 5 mL syringe, aspiring 2.5 mL from each eye and storing at 4 °C. VH [K^+] determined by direct potentiometry, [Hx] by HPLC, and [urea] by a point-to-point kinetic method	The equation PMI = 0.183Hx + 0.133U + 2.875[K^+] gave a 95%CI of ± 2.78 h. $R=0.769$ and 0.722 for [K^+] and [Hx] respectively	No significant differences in analyte concentrations between eyes. VH [K^+] gave a steep slope due to high Ta values. The sample was small
Garland et al. [42]	Retrospective cohort study, Australia	DV: VH [K^+], cerebral-CSF [K^+] and lumbar-CSF [K^+]. IV: PMI. To study the relationship between VH and CSF [K^+] and PMI by comparing univariate and multivariate regression models	$N=20$ samples from cadavers of medico-legal interest Samples from cadavers of people murdered or suspected of having been murdered were excluded, as were those from cadavers of children, of people who were submerged in water, and those with unknown PMI	Scleral puncture and aspiration of VH with a 20 G, 10-mL syringe. Heat pre-treatment of samples (100 °C, 5 min) followed by centrifugation (3000 rpm, 5 min). [K^+], [Na +], and [Cl-] determined by indirect potentiometry	VH [K^+] correlated best with PMI ($R=0.8$); the R values for lumbar-CSF and cerebral-CSF were much smaller (0.42 and 0.12 respectively). Using multivariate models, the improvement was marginal (not significant) for VH [K^+] and lumbar-CSF	VH [K^+] increased with PMI, while VH [Na +] and [Cl-] fell (although not significantly). Heat pre-treatment did not improve the accuracy of PMI estimates. Given the small sample size, the paper was exploratory in nature

Table 1 (continued)

Authors and date of publication	Study design and where performed	Dependent (DV) and independent (IV) variables and aims	Sample characteristics	VH extraction, pre-treatment, and analysis	Results and adjustments	Observations and comments
Focardi et al. [43]	Retrospective cohort study, Italy	DV: VH [K^+] and [albumin], IV: PMI. To determine whether a correlation exists between [K^+] + [albumin] and the PMI in a multiple regression model, and whether indirect potentiometry is as good as flame emission spectrophotometry for determining [K^+]	$N = 125$ samples from cadavers of medico-legal interest, all with known PMI (20–200 h) Samples from cadavers of people with eye trauma or eye disease, chronic kidney disease, other chronic diseases, large electrolyte alterations, who were dehydrated, or who had overdosed on drugs, were rejected	Scleral puncture using a 20 G, 10 mL syringe, followed by centrifugation (500 rpm, 10 min). [K^+], [Cl-] and [Na +] determined by indirect potentiometry, [Mg2 +] and creatinine by colorimetry, glucose, urea uric acid by enzymatic methods, and albumin by laser nephelometry	Linear relationship confirmed between [K^+] and PMI (95%CI ± 11.66 h), with no significant correlation between PMI and [Na+], [Cl-], [Mg2 +], or urea, uric acid, glucose or creatinine concentrations. The best multiple regression model for estimating PMI was $PMI = 5.35[K^+] + 9.94$ albumin—27.93 ($R = 0.84$). Indirect potentiometry (which is cheap and widely available) was just as good as flame emission spectrophotometry for determining [K^+]	VH [K^+] was the best predictor of PMI up to 72 h. [albumin] was better from 72–200 h—but there were few samples in this range. A multiple regression model with log([albumin]) instead of [albumin] increased the p value from < 0.05 to < 0.001, and the R value from 0.84 a 0.91)
Palacio et al. [44]	Retrospective cohort study, Italy	IV: VH [K^+] and [NH_4^+] DV: PMI. To examine whether the above analytes together are good markers of PMI, and to see whether capillary electrophoresis is a good method for determining these analytes	$N = 33$ samples from cadavers of medico-legal interest, all with known PMI, aged 19–75 years. Samples from cadavers of people with eye trauma or who had overdosed on opiates were excluded	Microscleral puncture (100–200 µ) and storage at − 24 °C (< 1 week). Analysis by capillary electrophoresis with UV light	In univariate models, the R values for [K^+] and [NH_4^+] were 0.75 and 0.7 respectively. When combined, the R value was 0.74. The estimated error for [K^+] and [NH_4^+] was ± 17.8 h and ± 20.2 h respectively. When the analytes were combined, it was ± 21.5. The correlation became stronger with increasing PMI	No difference between eyes. Although not better than [K^+], [NH_4^+] could be used as a complementary analyte in persons who died by opiate overdose or with trauma. Capillary electrophoresis was deemed adequate for determining these analytes

CSF, cerebrospinal fluid; *HPLC*, high-performance liquid chromatography; *Hx*, hypoxanthine; *LC–MS/MS*, liquid chromatography and mass spectrometry; *LS*, synovial fluid; *mEq/L*, milliequivalents/litre; *PMI*, post-mortem interval; *R*, linear coefficient of determination; *r*, Pearson's correlation coefficient; *SD*, standard deviation; *Ta*, ambient temperature; *Tr*, rectal temperature; *VH*, vitreous humour

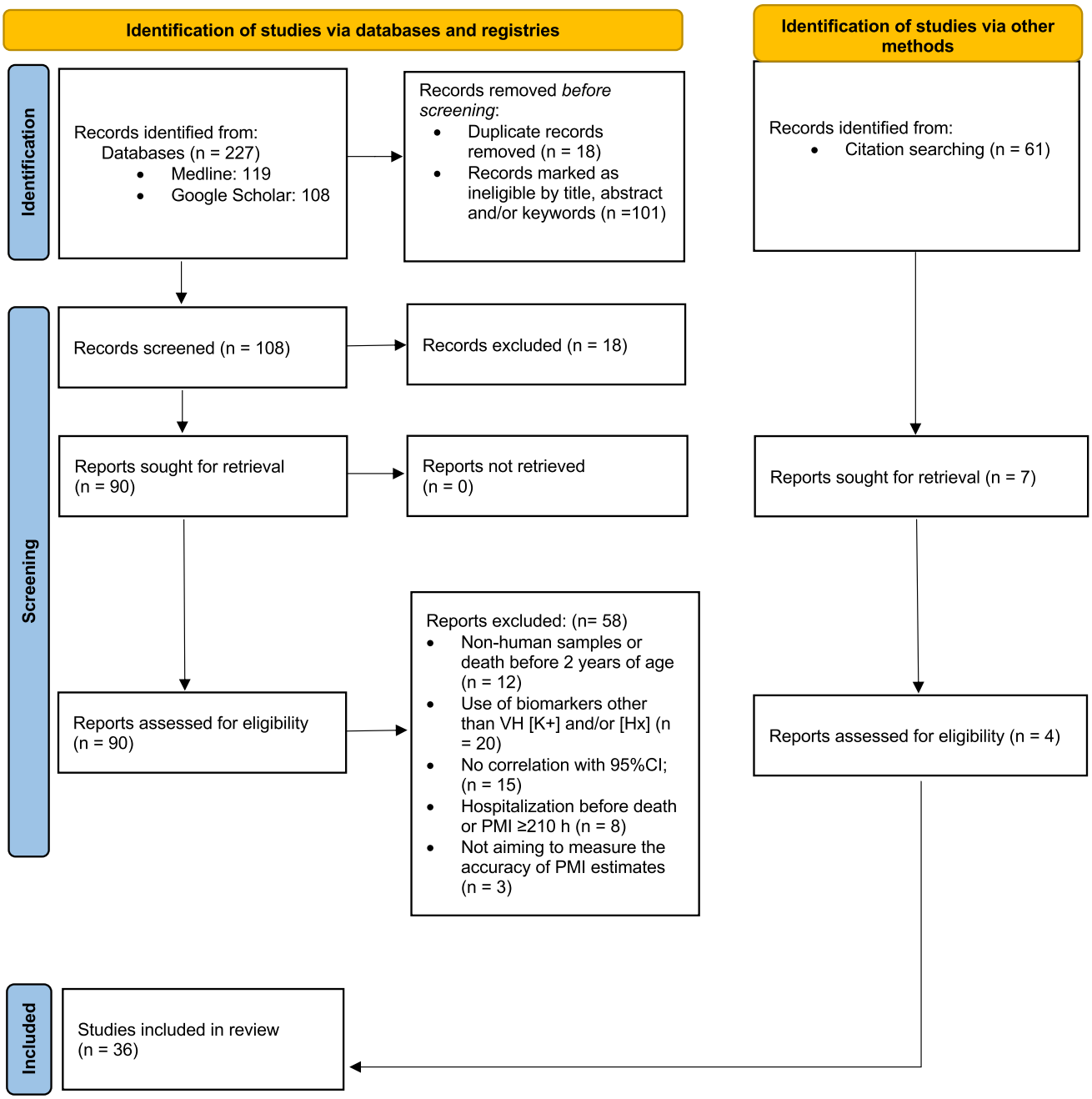

Fig. 1 PRISMA 2020 flow diagram for selecting systematic reviews including searches of databases, registries, and other sources

the DV (excluding Zhou et al. [13] and Bortolotti et al. [14]). Before 2001, all papers were published with the PMI as the IV (excluding Lange et al. [15]). Most studies excluded contaminated VH, and that from cadavers belonging to people with a chronic disease, who suffered electrolyte alterations, took diuretics, were dehydrated, or had chronic kidney disease. In four studies, those who had died from drug overdoses were excluded, and in three, victims of drowning were excluded.

Different treatments of the VH samples were noted. Sclera puncture with slow but complete aspiration, followed by centrifugation, was the most common pre-analytical step taken. Three papers used VH microsamples, while one involved repeated sclera puncturing. Studies also used different sample preservation methods, including the storage temperature used. Finally, in 17 studies, VH [K^+] was determined by direct or indirect electrode potentiometry, 12 used photometry, two used capillary electrography, one low-pressure ion

chromatography, and one liquid chromatography and mass spectrometry (LC–MS/MS).

Correlations between analytes and PMI

Of the 36 studies examined, 24 involved the use of simple linear regression to correlate VH [K^+] with the PMI (Table 2). Coe et al. [23] concluded that large variations existed in the error obtained (± 26 h), and that the Sturner et al. [3] model was the most accurate. In 1989, Madea et al. [24] obtained a 95%CI of ± 34 h with their model, improving to ± 22 h if samples with VH [urea] of > 100 mg/dL were excluded, and to ± 20 h if persons who suffered an agonal period of > 6 h were excluded. Later, in 1994, the same authors showed that VH [Hx] was no better than VH [K^+] for determining PMI, and that the increase in CSF [Hx] was not linear [27].

In 2001, using [K^+] as the IV, Muñoz et al. [6] obtained a better correlation between VH [K^+] and PMI, changing the established paradigm in which the latter is determined [14]. In 2005, Madea et al. [2] also concluded that the relationship between VH [K^+] and PMI is not entirely linear due to inter-individual variation. They also noted that multiple regression analysis improved the accuracy of PMI estimation.

The window during which increases in VH [K^+] with PMI were found to be linear differed between publications. Tatiya et al. [38] reported a linear increase until a PMI of 48 h. Zhou et al. [13], Bortolotti et al. [14], and Nermeen et al. [32] reported this linear relationship to be stronger in cadavers with a PMI of > 24 h.

In 2016, Ortmann et al. [36] concluded that in European climates the models of Madea et al. [29] and Muñoz et al. [6] provided the most accurate results, but that new formulae were needed for hotter climates. However, this conclusion

Table 2 Formulae for estimating the post-mortem interval

Study	PMI (h)	Formula
Sturner et al. [3]	104	PMI = 7.14 [K+] − 39.1
Adjutantis et al. [17]	12	PMI = 1.36 [K+] − 2.93
Aggarwal et al. [18]	35	PMI = 1.94 [K+] − 7.95
Devenaux et al. [19]	168	[K^+] = 0.124PMI + 7.60
Stephens et al. [21]	35	PMI = 0.258 [K+] + 6.342
Madea et al. [24]	130	PMI = 5.26 [K+] − 30.9
Rognum et al. [25]	120	$y = 4.2x + 7.6$ a 5 °C $y = 5.1x + 7.6$ a 10 °C $y = 6.2x + 7.6$ a 15 °C $y = 8.8x + 7.6$ a 23 °C
Gamero et al. [26]	24	[K+] = 0.158 PMI + 6.347
Madea et al. [27]	105	PMI = 6.31 [K+] + 0.179 PMI = 3.69 [Hx] + 1.29
Muñoz et al. [6]	29	PMI = 3.92 [K+] − 19.04
Muñoz et al. [9]	29	PMI = 3.631 [K+] − 17.344 PMI = 0.172[Hx] + 0.17
Zhou et al. [13]	27	[K+] = 0.1702 PMI + 5.56
Bortolotti et al. [14]	100	[K+] = 0.1733 PMI + 2.3
Mihailovic et al. [31]	30	PMI = 2.749 [K+] − 11.978
Nermeen et al. [32]	100	PMI = 8.77 [K+] − 53.90
Tumram et al. [33]	35.18	PMI = 2.71 [K+] − 20.19
Swain et al. [34]	96	PMI = 2.88 [K+] − 11.86
Rognum et al. [35]	60	PMI = 0.215[Hx] − 0.467T^a − 0.05[Hx]T^a + 10.35 PMI = 5.184 [K+] − 0.174T^a − 0.1[K+] T^a − 19.58
Tatiya et al. [38]	46	PMI = 0.204 [K+] + 9.63
Cordeiro et al. [7]	22.5	1) $\log(IMP) = \alpha + f_1(t^a\ rectal) + f_2(t^a\ environmental) + f_3(K^+) + f_4(Hx) + f_5(urea) + f_6(weight) + \varepsilon$ 2) $\log(IMP) = \alpha + f_1(t^a\ rectal) + f_3(K^+) + f_4(Hx) + f_5(Urea) + f_6(weight) + \varepsilon$
Saldaña et al. [41]	33	PMI = 0.183[Hx] + 0.133U + 2.87[K+]
Focardi et al. [43]	200	PMI = 5.35[K+] + 9.94Alb − 27.93
Palacio et al. [44]	168	$y = 0.0005x^2 + 0.2018x + 6.173$ for [K+]

does not take into account seasonal variations, sun exposure, shade, or indoor/outdoor conditions.

Devenaux et al. [19] concluded a positive significant linear correlation to exist between VH [K^+] and PMI, and a negative correlation between PMI and both VH [Na^+] and [Cl^-]. Other studies also focused on whether there was a linear correlation between combinations of analytes and PMI. VH [K^+] with [Na+] and [glucose] [25], and VH [K^+] and [NH_4^+] [43], as well as VH [Hx] with [lactic acid] in cadavers grouped by season of the year and Ta, were all examined [40], but VH [K^+] and [Hx] alone were found to be the most reliable. The reliability of VH and CSF analytes, and their combinations, was also examined; VH analytes alone were found to give the best results [42]. In these studies, VH [K^+] and VH [Hx] were both found to show a linear relationship with PMI, although this was contradicted by Chandrakanth et al. in 2012 [30].

Among the studies that involved non-linear, multivariate analysis, Lange et al. [15] used the Loess regression and obtained a smaller standard error with lower VH [K+] values. However, Madea et al. [29] determined the latter model to overestimate the PMI. In 2016, Rognum et al. [35] used two separate multivariate algorithms with [K^+] and [Hx]. In 2018, Srettabunyong et al. [39] correlated PMI with VH and synovial fluid [K^+], [Na^+], [glucose], [creatinine], [urea], [lactate], and [uric acid], and obtained good PMI results for both backgrounds. Other authors [14] worked with a multivariate model combining [Hx], [urea], and [K+], while Focardi [43], in 2020, developed a model combining [K+] and [albumin] or log[albumin] which had a very good fit. In 2018, Cordeiro et al. [7] concluded that generalized additive models were better for determining the PMI, and improved when physico-chemical variables were included. The model that combined Tr, Ta, and VH [K+], [Hx], [urea], and bodyweight gave the highest *R* value (0.786), while that which used Tr and VH [K+], [Hx], [urea], and bodyweight gave the lowest mean squared error.

Other factors that might modify the predictor variable-PMI relationship

In 2005, Madea et al. [2] reported that the slope correlating VH [K^+] and PMI should be steeper as Ta increases. According to Coe [23], the regression slope may vary between 0.14 and 0.55 mEq/L/h. As the PMI becomes longer, the diffusion velocity of K^+ becomes slower as equilibrium is approached. Muñoz et al. [6, 9] suggest that not properly stating which variable is the IV, and which is the DV, makes comparisons between studies difficult.

Many factors might affect the slope obtained: for example, the effects of electrolytic alterations before death on the predictor variable-PMI relationship, the duration of any disease, and the length of the agonal period. Many studies excluded cadavers with signs of eye trauma or chronic disease, with non-transparent VH, or that were victims of decapitation. Madea et al. [24] reported that knowing the urea concentration before death, and whether the agonal period was prolonged, significantly improved the accuracy of PMI estimates. Muñoz et al. [6] reported better accuracy in determining non-hospital death PMIs when the VH [urea] was > 30 mg/dL and [creatinine] ≤ 0.5 mg/dL (95%CI ± 17.55 h.

In some publications [21, 43], cadavers of people with sepsis or drug intoxication were excluded, and some involved only persons aged 18–65 years or with a PMI of 3–30 h. Tatiya et al. [38] and Cordeiro et al. [7] included cadavers with a PMI known to be within ± 15 min. The latter authors also excluded samples from people who died in a state of hypo-/hyperthermia but took data on any resuscitation manoeuvres used. Srettabunjong et al. [39] included very early samples (< 8 h) and excluded cadavers that had been submerged in water. Go et al. [40] classified cadavers with a PMI of < 103 h according to the season of the year in which death occurred. Garland et al. [42] ruled out cadavers from people who were murdered, those submerged in water, and the bodies of children. Finally, Palacio et al. [44] only examined cadavers of people aged 19–75 years and excluded those showing signs of opiate intoxication.

The sample sizes in each paper examined, how they were obtained, pre-treated, and analysed, may also have influenced PMI estimates (Table 1).

Finally, other variables of interest may be important. Stephens et al. [21] considered the cause of death to be a determining factor in PMI estimation. Lange et al. [15] affirmed that as VH [K^+] increases, so too does this error. This was later corroborated by Ortmann et al. [36].

Coe et al. [23] reported that the differences between eyes were more evident when determined by indirect potentiometry than by flame emission spectrophotometry, and that VH [K^+] was higher in children and in samples with VH [urea] > 100 mg/dL. They also recommended that as much VH be aspirated as possible so that the results were not affected by any difference in concentration between different parts of the vitreous chamber. Rognum et al. [25] showed the VH [Hx] to be elevated in children who had died from sudden infant death syndrome or infection. In contrast, in live patients undergoing vitrectomy, VH [Hx] was close to zero. It may thus be concluded that significant periods of hypoxia prior to death induce [Hx] elevation. It should be noted that [Hx] may be influenced by electrolyte alterations and alcoholism [35].

Gamero et al. [26] and Madea et al. [29] reported age to have a major influence on VH [K^+]. They also concluded the variation in VH [Hx], but not in CSF [Hx], to be linear. Mild and moderate hypoxias were found to significantly affect VH [Hx], as did electrolyte alterations, alcoholism,

and genetically related abnormalities in purine metabolism [35]. The formulae suggested by Rognum et al. [35]—with precautions and restraints—are available in an app (*iTOD*) available for free in app stores.

Muñoz et al. [6] indicated that cause of death and admission to hospital before death have a significant influence on PMI estimates. Age was also said to affect VH [K^+] (with greater concentrations seen in children). The same authors, as well as Saldaña et al. [41], also concluded that VH [urea] could be used as a pre-death marker of electrolyte balance since it changed little with advancing PMI.

Schmidt et al. [22] reported no differences between the two eyes when sampled < 4 h or > 4 h apart. Madea et al. [2] reported a 10% difference between the eyes when samples were analysed using different techniques. Other authors saw no such differences [14, 22, 30–32].

Cordeiro et al. [7] found neither age, cause of death, resuscitation manoeuvres, nor sex to have any significant influence on PMI estimates. They did conclude, however, that the post-collection storage temperature should be < 10 °C, and that bodyweight did influence PMI estimates. Srettabunjong et al. [39] reported that [K^+], [Na^+], [Cl^-], and [Mg^{2+}] were higher in the VH than in the synovial fluid. Finally, Coe et al. [23] determined the gold standard for VH collection to be scleral puncture with slow aspiration of all the VH possible using an 18-20G needle, followed by centrifugation at 3000 rpm for 10 min (with the freezing of samples not immediately tested). This procedure was the most commonly used in the papers examined.

Table 2 shows the great variation in the formulae used to estimate the PMI. These variations are the result of differences in the sample sizes available, the collection and pre-treatment of samples, and the analytical technique used to determine the analyte concentrations.

Methodological quality of the examined studies and their limitations

The present work examined three reviews of cohort studies and 33 retrospective cohort studies. The reviews of Coe et al. [23], Lange et al. [15] and Madea et al. [2] discussed papers with SIGN classifications [9] of 2 + + B. The retrospective cohort studies of Chandrakanth et al. [30], Srettabunjong et al. [39], and Garland et al. [42] were classified as 2- D, while those of Adjutantis et al. [17], Aggarwal et al. [18], Gamero et al. [26], Mulla et al. [28], and Palacio et al. [44] had classifications of 2- C. The remaining 25 studies were classified as 2 + C. Tables 3 and 4 show the results of the CASP analysis of the reviews/cohort studies [12].

Both Coe et al. [23] and Madea et al. [2] (Table 3) examined the results of quality papers (scores 9 and 8 out of 10 respectively). Lange et al. [15] used the data in their review

Table 3 CASP assessment of the reviews/cohort studies

Studies	Internal validity					Results		External validity			Total score
Items	Did the review address a clearly focused question?	Did the authors look for the right type of papers?	Do you think all important studies were included?	Did the review's authors do enough to assess the quality of the included studies?	If the results of the review were combined, was it reasonable to do so?	What are the overall results of the review?	How precise are the results?	Can the results be applied to the local population?	Were all important outcomes considered?	Are the benefits worth the harms and costs?	
Coe et al. [23]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	Yes	9
Lange et al. [15]	Yes	Yes	No	Yes	No	1	1	No	No	Yes	6
Madea et al. [2]	Yes	Yes	Yes	Yes	Yes	0	0	Yes	Yes	Yes	8

Table 4 CASP assessment of the retrospective cohort studies

Studies	Internal validity					Results		External validity			
Items	Did the study address a clearly focused issue?	Was the cohort recruited in an acceptable way?	Was the exposure and outcomes accurately measured to minimize bias?	Have the authors identified all important confounding factors?	Was the follow up of subjects complete and long enough?	What are the results of this study	How precise are the results?	Do you believe the results	Do the results of this study fit with other available evidence?	Can the results be applied to the local population?	Implications of this study may change clinical practice or result in a paradigm shift?
Sturner et al. [3]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Krause et al. [16]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	No
Adjutantis et al. [17]	Yes	Yes	Yes	No	Yes	1	1	Yes	No	Yes	No
Aggarwal et al. [18]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	No	No
Devenaux et al. [19]	Yes	Yes	Yes	No	Yes	1	1	Yes	Yes	Yes	No
Madea et al. [20]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Stephens et al. [21]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	Yes	No
Schmitdt et al. [22]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	No	Yes
Madea et al. [24]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Rognum et al. [25]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Gamero et al. [26]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	Yes	No
Madea et al. [27]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	No
Muñoz et al. [6]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Muñoz et al. [9]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Yes
Mulla et al. [28]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	No	No
Madea et al. [29]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	No
Zhou et al. [13]	Yes	Yes	Yes	Can't Tell	Yes	1	1	No	No	Can't Tell	No

Table 4 (continued)

Studies	Internal validity					Results		External validity			
Items	Did the study address a clearly focused issue?	Was the cohort recruited in an acceptable way?	Was the exposure and outcomes accurately measured to minimize bias?	Have the authors identified all important confounding factors?	Was the follow up of subjects complete and long enough?	What are the results of this study	How precise are the results?	Do you believe the results	Do the results of this study fit with other available evidence?	Can the results be applied to the local population?	Implications of this study may change clinical practice or result in a paradigm shift?
Bortolotti et al. [14]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Can't Tell
Chandra-kanth et al. [30]	Yes	Yes	Yes	No	Yes	0	0	No	No	No	No
Mihailovic et al. [31]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	No	Yes	No
Nermeen et al. [32]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	No	No	No
Tumram et al. [33]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	No	No
Swain et al. [34]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	No	No
Rognum et al. [35]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	No
Ortmann et al. [36]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	Yes	Yes
Tatiya et al. [38]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	No	No
Cordeiro et al. [7]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	No	Yes	Yes
Srettabun-jong et al. [39]	Yes	Yes	Yes	Yes	Yes	0	0	No	No	No	No
Go et al. [40]	Yes	Yes	Yes	Yes	Yes	1	0	Yes	Yes	No	No
Garland et al. [42]	Yes	Yes	Yes	Yes	Yes	1	1	No	Yes	No	No
Saldaña et al. [41]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	No	No
Focardi et al. [43]	Yes	Yes	Yes	Yes	Yes	1	1	Yes	Yes	Yes	Can't Tell

Table 4 (continued)

Studies	Internal validity					Results		External validity			
Items	Did the study address a clearly focused issue?	Was the cohort recruited in an acceptable way?	Was the exposure and outcomes accurately measured to minimize bias?	Have the authors identified all important confounding factors?	Was the follow up of subjects complete and long enough?	What are the results of this study	How precise are the results?	Do you believe the results	Do the results of this study fit with other available evidence?	Can the results be applied to the local population?	Implications of this study may change clinical practice or result in a paradigm shift?
Palacio et al. [44]	Yes	Yes	Yes	Yes	Yes	1	1	No	Yes	Yes	Can't Tell

to determine a model for estimating PMI by local regression. Referring to Lange et al. [15], Madea et al. [2, 24] indicate that the variation in VH [K^+] in six of the studies examined rendered results inapplicable to other populations. When Madea et al. [2] used the latter authors' regression model with their own data, they noticed several overestimates.

The retrospective cohort studies (Table 4) had notable limitations. For example, in the study of Sturner et al. [3], the burnt or frozen cadavers studied had no control group, and the formula presented only works if the Ta is < 10 °C (2), and even then, it tends to overestimate the PMI [36].

Some papers, such as that of Adjutantis et al. [17], only involved cadavers with a short PMI.

The formula of Aggarwal et al. [18] underestimated the PMI, as shown by Gamero et al. [26], and their samples only included a few cadavers with a PMI of < 24 h. Stephen et al. [21] only included cadavers with a PMI of up to 35 h.

Madea et al. [24] proposed an arbitrary VH [urea] limit of > 100 mg/dL. They did not examine any other markers of pre-death electrolytic alteration.

Rognum et al. [25] used repeated extractions, but the trauma generated may have increased the VH [Hx]. According to Madea [24], the latter authors could not properly conclude that the slope for VH [K^+] was steeper than that for [Hx].

To allow for comparisons between VH and synovial fluid analysts, Srettabunjong et al. [39] excluded cadavers of people with joint disease, and had no pre-death information for any cadaver, perhaps reducing the study's external validity.

Mihailovic et al. [31] did not take into account the effect of Ta on VH [K^+]. The repetitive sampling reduced the volume of the VH; this, plus the diffusion occurring, could have led to a further elevation of VH [K^+].

Tumram et al. [33] studied cadavers with a PMI of up to 18 h, but the regression slope obtained is only applicable in hot climates.

Chandrakanth et al. [30] examined cadavers with a maximum PMI of 36 h, some of which were victims of poisoning, and the study group ($n = 70$) and control group ($n = 30$) numbers were unbalanced.

Cordeiro et al. [7] log-transformed their values to ensure the homoscedasticity of their models—an inconvenience (although easily resolved).

Go et al. [40] did not consider the influence of the cause of death since they had no pre-death information. This is also apparent in other publications [16, 19, 22].

Saldaña et al. [41] worked with a very small sample and indicated that the analytical equipment they used had limitations.

The work of Garland et al. [42] was exploratory and involved just 20 samples. The effects of sample storage, Ta, and pre-treatment were not analysed, and PMI was defined

as the time between finding the body and the time of sample analysis.

Focardi et al. [43] included cadavers of PMI > 72 h but the effects of sex, age, and bodyweight were not studied.

Finally, Devenaux et al. [19], and Palacio et al. [44], had a small sample size, and the variation in the results and the standard error for cadavers with a PMI of < 60 h was large.

Estimating the PMI in Galicia

The IMELGA centres in Galicia examined 1913 cases. Those involving persons who had spent a prolonged period in hospital, and cases in which there was a question about the reliability of the information, were excluded, leaving a total of 1768 cases for study. The sample examined included 1256 men and 512 women. The mean number of deaths was 148.67/month (SD ± 17.8).

Forensic reports normally mention the major cause of death based on the International Classification [46], but this classification is insufficiently precise for a study of the present nature. The most common cause of death was cardiovascular (ischemic heart disease), followed by asphyxia (more specifically, hanging); see Fig. 2. The cause of death in 973 (55.03%) cases was natural, in 495 accidental, in 285 suicide, and in 15 (0.85%) homicide.

Table 5 shows the methods used. On very few occasions was PMI estimated using more than one method. Most often, it was estimated only from circumstantial evidence (54.75%). VH biochemistry was used on its own on 10 occasions, combined with cadaveric phenomena plus forensic entomology on three occasions, with cadaveric phenomena on two, and with temperature on another two. The formula most used was that of Muñoz et al. [4] (eight occasions), followed by that of Madea et al. [18] (four occasions). Figure 3 shows that quantitative methods were only used in the first 72 h after death; cadaveric phenomena were most commonly employed to determine the PMI (93.73%).

The Galician data show that circumstantial information (such as witness' accounts) is commonly deemed sufficient to estimate the PMI. It is more easily obtained and more reliable when less time has elapsed since death. This is reflected in the low mean PMI value and small associated

Table 5 Descriptive analysis of PMI estimation methods from our database ($n = 1768$)

Method	n_i	N_i	f_i	F_i
CE	968	968	0.5475	0.5475
CP	700	1668	0.3959	0.9434
CE + CP	40	1708	0.0227	0.9661
T^a	34	1742	0.0193	0.9854
VH	10	1752	0.0057	0.9911
T^a + CP	5	1757	0.0028	0.9939
VH + CP + FE	3	1760	0.0017	0.9956
FE + CP	2	1762	0.0011	0.9967
T^a + VH	2	1764	0.0011	0.9978
T^a + CE	2	1766	0.0011	0.9989
VH + CP	2	1768	0.0011	1.0000

CE, circumstantial evidence; *CP*, cadaveric phenomena; *FE*, forensic entomology; T^a, temperature; *VH*, vitreous humour biochemistry; n_i, absolute frequency; N_i, cumulative absolute frequency; f_i, relative frequency; F_i, cumulative relative frequency

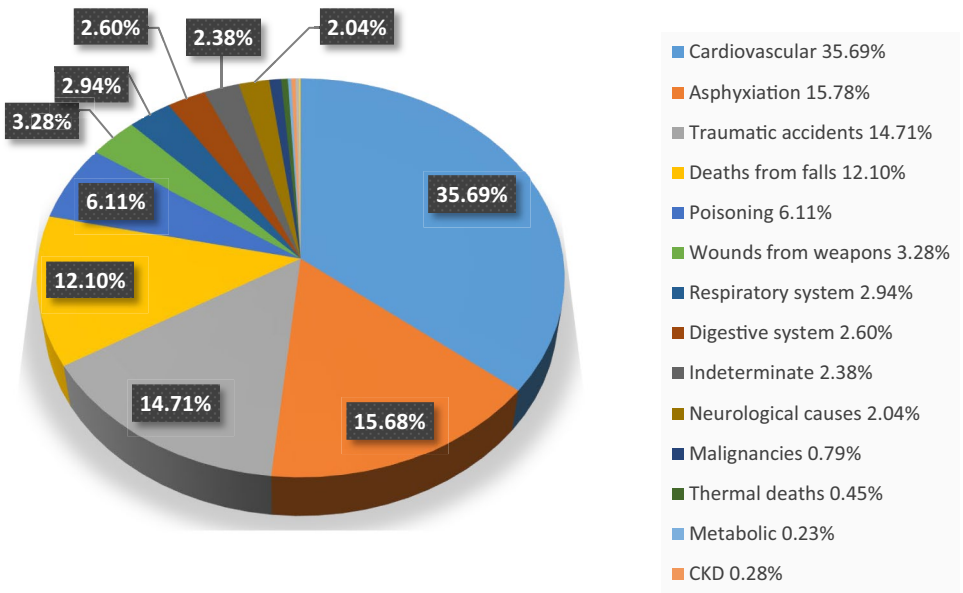

Fig. 2 Causes of death according to the International Classification of Diseases

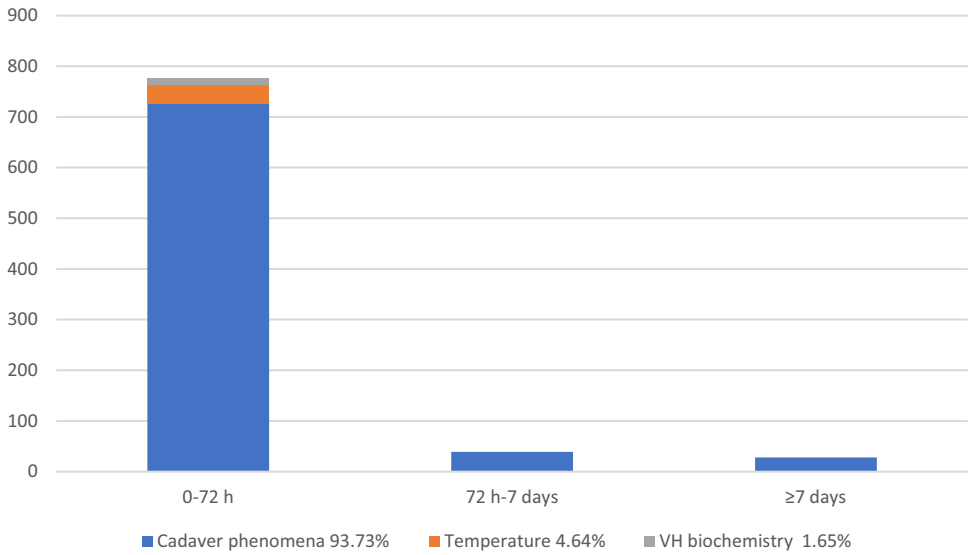

Fig. 3 PMI estimation methods performance according to time of death

SD (Table 6). In cases with late cadaveric phenomena and signs of putrefaction, the method used to determine the PMI relied on these same phenomena; VH biochemistry would not be reliable in such cases, leaving any analytical results without value. In cases in which an intermediate interval had elapsed since death, biochemical methods and Tr were used to estimate the PMI. It is therefore uncertain whether taking just one sample for testing via only one method is good enough for estimating the PMI. In practice, two or more samples should be taken and more than one method used to estimate the PMI (although this is not always possible). Despite the impossibility of providing a statistical analysis, the present data doubtlessly open the debate on the need to use more than one method to estimate PMI.

Table 6 Methods used to determine the PMI

Method	Mean PMI (h)	Standard deviation (h)	Coefficient of variation
Circumstantial evidence	2.323	7.069	3.043
Cadaveric phenomena	39.211	229.861	5.862
VH biochemistry alone	6.581	9.120	1.386
Temperature (*Henssge*)	7.720	8.852	1.147
Combination temperature and VH biochemistry	1.345	4.153	3.088
Combinations without temperature and VH biochemistry	36.000	164.992	4.583
Overall	7.716	32.403	2.209

Discussion

Numerous methods are available for correlating VH with the PMI. VH [K+] and the PMI are best related via the formulae of Madea et al. [24] or Muñoz et al. [6, 9]. Ta, which has been reported to strongly influence VH [K^+] and [Hx] [35, 36], was omitted from many models. A correlation has also been recorded between the increase in VH [K^+] and [Hx]. The values for VH [Hx] in the first 24 h after death are, in fact, less dispersed than those for VH [K^+], but PMI estimates are less accurate if determined from VH [Hx] alone than from VH [K^+] alone [2, 6, 24, 35].

PMI estimates are influenced by variables such as trauma, eye disease, and hydroelectrolytic disturbances [6, 24]. These factors were considered in most of the studies examined.

High values for VH [urea] and [creatinine] have been related to higher VH [K+] [6, 36], although few studies took this into account. Age, however, may not be important except when the cadaver is that of a very young child [7, 27]. Finally, deaths by carbonization, freezing, or drowning were excluded in most studies.

Despite the linear trend in VH [K^+] and [Hx] regarding PMI, the slope was somewhat steeper in the first 6 h [23]. This trend was seen in most papers, although Chandrakanth et al. [30] found no correlation between [Na^+], [K^+], [Cl^-], or [Na^+]/[K^+] and PMI. This contradiction might be explained in that the latter authors only included cadavers with a PMI of up to 36 h, death by poisoning was not excluded, and the study and control groups were of different sizes and PMI.

Lange et al. [15], using Loess local regression, indicated the increase in VH [K+] not to be linear. However, Madea et al. [2] report that the latter authors' findings not to have

been borne out in independent studies because they only used a Loess curve with a single 95%CI to explain the trends of six large studies covering a wide range of VH [K+]. When applying the same model to their own data, Madea et al. [2] reported it to return many overestimates.

Most papers showed strong correlations in linear regression. It might be thought that any formula might work. However, the same data cannot be used to construct a model and then used to test it [10], and many studies made this mistake. This leads to results that appear better than they really are. Independent datasets are needed to validate a model. However, a Monte Carlo–like analysis can be made. In the studies examined, Cordeiro et al. [7] were the only authors to do this. Generalized additive models carry more weight and improve the 95%CI [45, 47, 48]. The disadvantage is that, being additive, the influence of certain interactions might be lost, making it important to adjust the weight of each variable for K_i correction factors [47].

Discussion continues as to which is the best analytical technique. In 2012, Lendoiro et al. [49] affirmed that HPLC was the best for determining [Hx]. Any pre-treatment of the VH samples should be done when they are fresh; when not possible, samples should be frozen. Frozen samples should be thawed in a refrigerator and then brought to room temperature for analysis; exposure to light should be avoided [50].

The classic technique for measuring VH [K+] is flame emission spectrophotometry. Many studies have mentioned significant differences between the left and right eye when this analyte is measured by indirect or direct potentiometry [13, 23, 28, 51]. New techniques being investigated include capillary electrophoresis with microsamples, which does not have the latter limitations and appears promising [2, 43, 44]. Low-pressure ion chromatography is cheap and widely available and would also appear to be adequate, but further studies are needed [38].

Other analytical tools and sampling methods for VH have also been developed. Risoluti et al. [52] reported thermogravimetric and chemometric techniques to be very accurate when used with either clear or contaminated VH. In addition, they indicated their method, which is claimed requires only a few microlitres of VH and needs no sample pretreatment, to be capable of predicting PMI to within an hour for recent cadavers. Following this line, they went on to develop a "two-way multiparametric platform" based on inductively coupled plasma–optical emission spectrometry (ICP–OES) and thermogravimetric analysis (TGA), and suggested the P, S, Mg, and the water contents of VH to predict PMI just as well as [K^+]; they even reported the range over which estimates could be made as up to 15 days [53]. In another work, Aiello et al. used a matrix-assisted laser desorption mass spectrometry–based method, and an untargeted metabolomics method, to analyse the VH. Using two multivariate statistical strategies (principal component regression and partial least squares regression), they reported $R^2 = 0.95$ for both methods, and a prediction error of 6 h for each when the PMI was < 24 h [54].

Although samples can be collected in different ways, this can lead to large biases and results that differ from what might be expected according to the literature [30]. Nevertheless, obtaining good VH samples becomes difficult when the PMI is more than 30–48 h.

Quantitative methods are rarely used in forensic cases in Galicia as information primarily comes from circumstantial data and cadaveric phenomena. Police and witness information was essential in most of the suspicious cases. PMI was either known or easily determined without the need to use analytical techniques. This might be due to the slow and complex nature of such techniques, a lack of experience in their use, or knowledge of their existence. In most cases, only one method was used to estimate PMI, but, in practical forensic casework, all collectable evidence should be included so that reliable conclusions can be drawn.

To increase the use of quantitative methods, it is necessary to train forensic experts, and systematize data collection. Predictive simple software tools are also needed if the use of quantitative methods is to be promoted.

Conclusions

VH [K+] and [Hx] are good markers for estimating PMI. The disparity in the results in the different papers examined, and the validity of the conclusions with respect to the estimation of PMI, is owed to:

1. The effect of Ta, drugs, differences between the right and left eyes, age, and bodyweight.
2. The use—or not—of gentle puncturing to prevent trauma during sample collection, of adequate pre-treatment, and whether samples were preserved by freezing if they could not be immediately tested.
3. The use of different analytical techniques for the determination of VH [analytes].
4. The non-uniform distribution of known PMIs, and the small number of samples for each PMI time range (especially those > 72 h).
5. The use of different informative variables

VH biochemistry alongside other physical factors provides the best means of establishing a PMI with acceptable error. Advanced regression models provide the most reliable formulae. Cross-validation is, however, needed to validate any model. The search for the "ultimate" means of estimating the PMI continues; proteomics and chemometrics methods might allow a more accurate and faster analysis of VH samples in the future.

In Galicia, most deaths of medico-legal interest were determined to be of cardiovascular origin, followed by asphyxia and traumatic accidents. Quantitative methods were not frequently used to determine the PMI; in most cases, only circumstantial evidence was used, although cadaveric phenomena were often relied upon. If VH biochemistry is to be used in the estimation of PMI, trained personnel, better storage for biological samples, and simple software tools will all be needed. Finally, the present work suggests that the cause of death categories employed by the International Classification of Diseases are too wide to be of use in the forensic research setting.

Key points

This paper provides:

1. A systematic review following PRISMA recommendations.
2. A scientific review of the biochemistry of the vitreous humour and its relationship with the post-mortem interval (PMI).
3. An assessment of the quality of the papers examined using the CASP tool.
4. New perspectives on predictive models for estimating the PMI via VH analyte concentrations and other variables.
5. Past and current analytical methods and regression models for estimating the PMI.
6. A descriptive study on the use made of PMI estimation methods in real forensic practice in Galicia.

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Declarations

Ethics approval The study was approved by the ethics committee of the University of Santiago de Compostela (USC-19/2020) and was carried out in compliance with the legal and ethical requirements (Spanish Acts 14/2007 and 3/2018).

Conflict of interest The authors declare no competing interests.

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